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(54) Title: ANTIBACTERIAL TREATMENT OF OSTEOARTHRITIS

(57) Abstract: The present invention relates to use of an antibacterial agent in the manufacture of a medicament for the treatment of osteoarthritis, more particularly for the treatment of a bacterial infection which is responsible for osteoarthritis. Also described are methods for the diagnosis of osteoarthritis through the detection of certain bacteria in an affected joint of a patient with osteoarthritis.

ANTIBACTERIAL TREATMENT OF OSTEOARTHRITIS

The present invention relates to osteoarthritis and methods for the treatment and diagnosis thereof.

Osteoarthritis is also known as 'degenerative joint disease' and is the most common type of arthritis, affecting an estimated 20.7 million adults in the United States of America alone (data from www.nih.gov/niams/healthinfo/artrheu.htm). Osteoarthritis primarily affects cartilage, the tissue that cushions the ends of bones within a joint. Osteoarthritis occurs when cartilage begins to fray, wear, and decay. In extreme cases, the cartilage may wear away leaving a bone-on-bone joint. Osteoarthritis (OA) can cause joint pain, reduced joint mobility and disability. Disability results most often when the disease affects the spine and the weight-bearing joints (the knees and hips).

Typically, the symptoms of OA develop rather slowly. A particular joint may ache or be sore after prolonged use or after a period of inactivity, e.g. sleeping. The pain, stiffness and soreness will generally get worse with time and the range of motion at the joint may become reduced. A grating sound on movement indicates that the cartilage in the joint has worn away and the bones are rubbing against each other.

Osteoarthritis is known as 'degenerative arthritis' and it may affect the knee, hip, spine and other structures; it is the most common of all human joint disorders. OA is classified as a non-inflammatory arthritis and thus can be distinguished from rheumatic diseases like rheumatoid arthritis which is classified as an inflammatory arthritis. Rheumatoid arthritis is an inflammatory disease of the lining of the joint

(synovium), it is associated with swelling and inflammation of certain joints, muscle pain and eventually the loss of use of the joint altogether. The inflammation tends to be symmetrical which helps in the diagnosis of rheumatoid arthritis. Other diseases within the group of rheumatic diseases include gout which most commonly affects the big toe and develops quickly, infectious arthritis which is a general term used to describe the various forms of arthritis caused by infectious agents such as bacteria or viruses, and reactive arthritis which develops after an infection involving the urinary tract, bowel and other organs and is often associated with eye problems, skin rashes and mouth sores.

The term 'arthritis' is sometimes used to refer to all rheumatic diseases, however the word literally means joint inflammation, i.e. swelling, redness, heat and pain caused by tissue injury or disease in the joint. The different types of arthritis comprise just a part of the rheumatic diseases which also includes diseases described as 'connective tissue diseases' and autoimmune diseases such as fibromyalgia and systemic lupus erythematosus. As discussed above, OA is no longer seen as part of this group as it is a non-inflammatory arthritis.

The cause of OA is not known but it is believed that it results from a combination of factors. It has been thought that increasing age, a family history of the disease, overuse or abuse of a particular joint, injury, being overweight and other diseases may all contribute to the development of OA. The cause or causes of types of infectious arthritis (bacteria and viruses) and gout (uric acid crystals in the joint) are better understood by scientists and clinicians.

The role of certain endogenously produced enzymes in the breakdown of joint cartilage in OA and the possibility of using drugs that block the action of

these enzymes has been investigated. In particular, nitric oxide, which is produced by a family of enzymes called nitric oxide synthases, is spontaneously released from human cartilage affected by OA in quantities sufficient to cause cartilage damage. There is a hypothesis that NO inhibits matrix production by interfering with important autocrine and paracrine factors and NO has been shown to inhibit the production of TGF-β. Activated articular chondrocytes produce large amounts of NO, and there is increasing evidence that this could be involved in the ethiopathogenesis of osteoarthritis. Because of its short half-life, the biological effects of endogenously produced NO are likely to occur locally within the cartilage. (R. Studer, Osteoarthritis and Cartilage Vol. 7, No 4, July 1999). Also, an important component of cartilage is Type II collagen which is degraded by the endogenous metaloproteinase, gelatinase.

Diagnosing rheumatic diseases or OA can be difficult because some symptoms are common to many different diseases. Diagnosis may require referral to a rheumatologist, as even if diagnosis of one of the rheumatic diseases has been made, it may require a specialist to determine which one.

Typically, a diagnosis will require a full review of the patient's medical history including family history, a physical examination, laboratory tests and X-ray or other imaging techniques. The physical examination will typically include investigation of all joints for redress, warmth, deformity, ease of movement and tenderness. As some forms of arthritis, such as lupus, may affect other organs, a complete physical examination including the heart, lungs, abdomen, nervous system, eyes, ears and throat may be necessary. Blood, urine and/or synovial fluid may be needed to perform one of a number of laboratory tests including: for antinuclear antibody, complete blood count, a

hematocrit, for rheumatoid factor and urinalysis.

The doctor may need to see the patient more than once in order to make the diagnosis and the final decision will generally be a working diagnosis based on a number of different parameters which relies on the competence and relevant experience of the examining practitioner. A less subjective test for OA which was quick and easy to perform would greatly improve the diagnostic process. As OA is common, it would be useful to identify OA positively but equally if OA could be ruled out at an early stage, this would also be of considerable benefit to the practitioner.

So far as currently available treatments for OA are concerned, there is no single, successful treatment available for all patients. A typical treatment plan usually combines several types of treatment depending on the stage and severity of the condition and the medical and lifestyle needs of the patient. Treatments may include rest and relaxation, exercise, diet changes and medication, in severe cases surgery may be necessary.

It is generally acknowledged (www.nih.gob/niams/healthinfo/artrheu.htm) that available medications used to treat most rheumatic diseases and OA do not provide a cure but rather limit the symptoms of the disease. Although infectious arthritis (e.g. Lyme disease), if diagnosed in time, can be successfully treated with antibiotics.

Medications commonly used to treat OA provide relief from pain. Suitable analgesics include aspirin and other non-steroidal anti-inflammatory drugs (NSAID's) such as ibuprofen, (which have the added benefit of decreasing the inflammation associated with tissue damage). In recent years, early changes in joints have been surgically treated by using a combination of cultivated cartilage cells and periostal covering from the patient, in an attempt to repair the damaged cartilage. Only a small number of patients can

be treated by this expensive and invasive method.

It has been postulated (Amin, A.R. et al. (1996) Proc. Natl. Acad. Sci. USA; 93, pp 14014-14019) that tetracyclines could be used in the treatment of OA because of their ability to inhibit the expression of endogenous nitric oxide synthase (NOS). The possibility of using the tetracycline doxycline in the treatment of OA because of its ability to inhibit the patient's collagenase activity was discussed by Yu, L.P. et al. (1991) in J. Rheumatol; 18, pp 1450-2. No product based on these compounds has yet made it on to the market.

It is clear from the above discussion that existing methods for the diagnosis and treatment of OA are not fully satisfactory. Given the number of sufferers of OA, particularly in the ageing western populations, there is a real need for quick and reliable diagnosis of OA and for improved treatments therefor. Following on from a new and surprising discovery, the present invention provides teaching which address both of these problems.

The present inventor has, for the first time, established a link between bacteria and osteoarthritis. It has never before been suggested that bacteria could have a primary role in OA and identification of this role opens up the possibility for a variety of new therapeutic and diagnostic techniques in the field of OA care. According to one aspect, the present invention therefore provides the use of an antibacterial agent in the manufacture of a medicament for the treatment of OA, more particularly for the treatment of a bacterial infection which is responsible for OA. Alternatively expressed, the present invention provides an antibacterial agent for use in the treatment of OA, more particularly for use in the treatment of a bacterial infection which is responsible for OA.

The symptoms and diagnosis of OA are discussed above and although it may be a complex process, a

suitably experienced practitioner is usually able to diagnose OA successfully. A useful definition of OA for the purposes of the present invention is as follows: pain in one or more joints, gradual wear of the cartilage in that joint (typically over several years), plus no signs of arthritic changes as are found, for example in rheumatoid arthritis.

Typical changes found in x-ray pictures from patients with OA include a narrow joint space and subchondrial sclerosis. OA is characterised by joint pain and loss of function caused by a generally progressive loss of articular cartilage, followed by attempted repair of the articular cartilage and also remodelling and sclerosis of subchondral bone. Subchondral bone cysts and osteophytes and secondary synovitis inside the joint may also be found. In contrast with the rheumatic diseases, inflammation is not a main aspect of the disease.

OA may be divided into primary and secondary OA, the present invention being of use in the treatment and diagnosis of both forms, particularly primary osteoarthritis. Primary OA is the more common form and occurs with increasing prevalence with increasing age, there is no currently available cure. A definition of primary OA is provided by the American College of Rheumatology (ACR) http://www.rheumatology.org/patients/factsheets.html as follows: OA is a heterogenous group of conditions that lead to joint symptoms and signs that are associated with defective integrity of articulate cartilage, in addition to related changes in the underlying bone and joint margins. Diagnosis will typically involve assessment of pathological, radiographical and clinical aspects.

Secondary OA is less common and causes include metabolic, developmental and genetic abnormalities of articular cartilage. In secondary OA a clear causal

condition, event or disease is recognised. Secondary OA often occurs in the following circumstances Stickler's Syndrome (progressive Hereditary Arthro-ophthalmopathy), hemochromatosis, calcium pyrophosphate deposition disease, articular surface injury, joint instability, joint incongruity, denervation (Charcot Joint), epiphyseal dysplasias, following septic arthritis (infection), osteonecrosis, osteochondritis dissecans and years after menisceal damage and/or menisceal removal.

In fact, the data presented herein regarding the role of bacterial infection in the development of OA could lead to its classification as a secondary condition, resulting from initial bacterial infection.

As discussed above, the present invention provides medicaments for use in the treatment of bacterial infections responsible for OA. The inventors are the first to identify a primary (i.e. causal) role for bacteria in OA, i.e. infection may be a primary event or secondary to an initiating event in OA but is nevertheless responsible for one or more (typically most or all) of the characteristic symptoms of OA and/or for exacerbating such symptoms. The term 'responsible for' should therefore be interpreted with this relationship in mind.

The clinical data presented herein regarding the treatment of osteoarthritic patients with an antibiotic confirms the molecular data and demonstrates a role for bacteria in osteoarthritis. The majority of patients tested were considered to show improved symptoms following just 4 weeks of treatment with an antibiotic.

Although not wishing to be bound by theory, it seems likely that there may be some initial damage to the cartilage and the OA-causing infection is transmitted through the synovial fluid or through blood vessels developed around the injured cartilage. This active role for bacteria in the development of OA can be

contrasted with secondary bacterial infections that may occur e.g. post surgery and are not themselves responsible for the symptoms of OA.

For example, antibiotics have been described previously in a prophylactic context (Espehaug, B. et al. (1997) J. Bone Joint Surg. Br. July; 79(4) pp 590-5). In this study antibiotic-containing cement and/or systemic antibiotics were given to osteoarthritic patients who had just received primary cemented total hip replacements. Implanting metal during the treatment of fractures or OA means increasing the risk of infection as bacteria may easily cruciate the area around a metal implant. The use of antibiotics in this context is not, however, for the treatment of a bacterial infection having a causal role in OA itself. By contrast, according to the present invention, the treatments is typically not prophylactic, i.e. an established infection which is responsible for the observed OA is treated. In addition, the particular bacteria which may cause post-operative problems are different from those now identified to have a causal role in OA and the symptoms thereof.

The present invention can also be distinguished over publications such as Amin et al. (supra) which suggest a possible role for tetracyclines in the treatment of OA through the ability of such molecules to inhibit endogenous enzymes including NOS and collagenase. Clearly, such documents are not concerned with and do not describe the use of tetracyclines in the treatment of a bacterial infection, their antibiotic activity being incidental to the activities of interest. There is no suggestion in these documents of a causal role for bacteria in OA.

The inventors have been able to make the link between bacteria and OA through a series of experiments. Previously, no bacteria have been found in joints with primary osteoarthritis (except in cases with exchange

surgery after loosening). As discussed above, sceptic (infectious) arthritis is a different condition and bacteria such as *Streptococcus* and *Staphylococcus* and some gram negative species are normally found in such joints. Only a fortuitous combination of techniques, namely the manner of biopsy taking, modified differential display methods and finally 16s rRNA analysis alerted the inventors to the possibility of bacterial involvement and then confirmed it. It is postulated that the presence of bacteria may have been 'hidden' in the past because the relevant bacteria are difficult to grow in standard culture tests.

The patients included in the "osteoarthritic" group were all going through arthroplasty procedures in a knee. The biopsies were taken during surgery when the joint was being replaced both on the femoral and tibial side. The diagnosis of osteoarthritis was based on pain in the joint, a typical gradual wear of the cartilage for years, no signs of arthritic changes as found in rheumatoid arthritis, and typical changes found on x-ray pictures like a narrow joint space and subchondral sclerosis. The pathological changes were far advanced; this is supported by the need for the patients to undergo joint arthroplasty.

One of the control groups included were patients with normal cartilage, from whom samples were taken during replacement of the anterior cruciate ligament. No recent trauma had disturbed the knees undergoing this operation and the piece of cartilage studied had to be removed during the replacement procedure to allow enough space for the repaired ligament to function. None of these control patients were found to have the identifying bacterial gene sequence.

In the other control group, patients had only localized damaged areas with no general damage to the joint. All were operated on in an attempt to regain the architecture and functions of normal cartilage. This

operation included the use of *in vitro* grown chondrocytes, removal of the damaged cartilage and the covering of the area to be repaired with periosteum and the *in vitro* grown cells. None of the patients with this limited damage of cartilage were found to have the bacterial gene. These results suggest that in very early stages of OA development there may be little or no bacterial involvement but that a bacterial infection is responsible for the development of full-blown OA and for the advanced and more serious symptoms thereof. It is the progression to full-blown (advanced stage) OA at a given joint which is responsible for the pain and lack of mobility experienced by most patients with OA and therefore treatments which prevent or slow this progression would be desirable.

Symptoms characteristic of early stage OA include pain during activity and a reduced level of activity. At an advanced stage of OA, pain may be observed at rest and at night, as well as during activity. Activity is very reduced and the use of crutches is common. Also associated with advanced OA are reduced movement of the joint and stiffness in the joint.

From the clinician's point of view, in the early stages of OA, joint line pain is found and there may be increased synovial fluid in the joint. Radiographs generally indicate a narrowing of the joint space because the cartilage is reduced. However, MRI scans are more useful than x-ray images in the early stages as they can identify early changes in the cartilage and subchondral bone. Arthroscopy may reveal fibrillation, cracks and defects in the cartilage. In advanced OA, there is increased joint line pain, palpation of osteophytes and usually increased synovial fluid. Radiographs reveal a significant narrowing of the joint space to the joint where it appears like bone on bone with no space for cartilage. X-ray also reveals sclerosis and cysts in the subcondral bone, possible

deformation of the joint and usually osteophytes around the joint margins. Arthroscopy reveals absent and degraded cartilage.

Thus, in a further aspect, the present invention provides the use of an antibacterial agent in the manufacture of a medicament for preventing or reducing the development of, or progression to, advanced stage osteoarthritis. If OA is diagnosed at a very early stage, then prophylactic treatment with an antibiotic may be appropriate. Alternatively expressed, the invention provides an antibacterial agent for use in preventing or reducing the development of, or progression to, advanced stage osteoarthritis.

A modified differential display technique was used which is described in detail in the Examples section herein. Differential display is a method which is used to discover genes that are differentially expressed in one situation compared to another. mRNA is reverse transcribed and from the cDNA population a small number of genes are amplified using selected primers and PCR. Separation of the samples under investigation (amplified with the same primer sets) side by side on a high resolution gel yields a pattern of bands, each representing one expressed gene in the original samples. A gene/band that is found in one sample but not in another is said to be differentially expressed.

In this case, biopsies were taken from the knee, in OA patients from the osteoarthritic part of the cartilage and from another patient during repair of the cruciate ligament. In one patient with OA, biopsies from both the osteoarthritic area and an undisturbed area were compared. There was a problem with insufficient mRNA in the cartilage sample. Cartilage tissue is built up of chondrocyte cells surrounded by extracellular matrix. The total number of cells per gram of tissue is low. The extracellular matrix consists mainly of collagen (type II, IX, XI), proteoglycans (aggrecan) and

other large molecules such as hyaluronan. Extracting mRNA from small cartilage samples yields almost undetectable levels of mRNA. This is both due to the low number of cells in a small piece of tissue with few cells per weight unit and to the effect of the extracellular matrix on the efficiency of the mRNA extractions/isolation. Proteoglycans readily bind to RNA and thus lowers the yield. Therefore the complete cDNA population was amplified as a preliminary step after reverse transcription.

The differentially expressed bands on the gel can be cut out, reamplified, cloned and then sequenced. This work identified NOS as more highly expressed in osteoarthritic cartilage and sequence analysis indicated that the gene had a greater homology with a bacterial sequence than a human sequence. The presence of bacteria was then confirmed by detecting 16S rRNA in osteoarthritic tissues, indicative of pathogenic bacteria in the affected cartilage tissue.

Further sequencing and comparative studies of the 16S rRNA has enabled identification of the bacterial species involved in OA, and this will allow selection of the most appropriate antibiotics. The species concerned is or is very closely related to *Janthinobacterium*. Duganella is an example of a species very closely related to *Janthinobacterium*. The sequence data indicates that the responsible bacteria is related to *Pseudomonas* sub-species. Such bacteria are different from the bacteria which cause problems in hospitals due to infection of wounds and surgical sites. There may be a heterogeneous bacterial population causing OA in the joint(s) of some patients.

By 'antibacterial agent' is meant any compound or formulation which kills bacteria, prevents or inhibits proliferation of bacteria or otherwise weakens or disables bacteria. Both bactericidal and bacteriostatic agents may be used. The agent may have a specific

activity for only one or a small number of bacterial species or it may be active against a broad range of bacteria, such as bacterial membrane affecting peptides.

Suitable antibacterial agents can be used locally or administered intravenously or orally. Treatment could include one or more of the following:

a- A small arthroscopy operation that will include flushing through the joint and injecting the selected antibiotic, or an arthrotomy with a needle injecting the selected antibiotic.

b- Again by arthroscopy, placing antibiotic-releasing matrix into affected joints.

c- Oral administration of a selected antibiotic combination.

d- Intravenous administration of a selected antibiotic combination.

Also, prosthesis or cells could be impregnated or formulated with antibiotics before implantation.

Many antibacterial agents are known and more are being developed all the time. Preferred antibacterial agents are those which are effective in the treatment of *Pseudomonas* infections. Antibiotics which are active even against bacterial species which have a tendency to form biofilms by quorum sensing are preferred, as *Pseudomonas*, *Janthinobacterium* and *Burkholderia* have a high tendency to form such biofilms. Thus, a preferred class of antibiotics are those which are targeted /against the genes or gene products involved in quorum sensing and biofilm formation.

Indeed, it has recently been shown that a major component in bio-films produced by *Pseudomonas* is DNA (the bacteria believed to be responsible for osteoarthritis are similar to *Pseudomonas*). Therefore in a preferred embodiment of the present invention, treatment will be a combined therapy where an antibiotic is co-administered with an agent which can break down DNA, preferably an enzyme e.g. a restriction enzyme or

DNase I. A synergistic effect occurs as the enzyme or other agent breaks down the DNA and reduces the viscosity of the bio-film enabling more effective penetration of the antibiotic. Anti-DNA agents such as DNase I will preferably be injected into the affected joint.

The following are specific examples of suitable antibacterial agents: clariththromycin/levofloxacin combination, mercaptoethylguanidine (has been found to inhibit the inflammatory response of *Pseudomonas* infection due to nitric oxide), ciprofloxacinlactate (effective against *Pseudomonas*), tobramycin (has been used against *Pseudomonas*), ceftazidimpentathydrate (alone or in combination with other antibiotics), gentamicin (is used locally in orthopaedic surgery for the treatment of infections and could be used in local treatment of osteoarthritis), ciproxin, rifampicin (in combination with ceftazidimpentathydrate and/or gentamicin), doxycycline (a broad-spectrum antibiotic) and trimetroprim/sulfamethoxazole (combination).

In some cases, for example when there is concern about side-effects following a systemic treatment, the antibacterial agent will preferably be injected into (or near) the affected osteoarthritic joint. Antibacterial agents in this category include gentamicin and Ciproxin. According to a preferred treatment regimen, 2 or more antibacterial agents will be co-administered e.g. gentamicin and doxycycline. In this case one active agent may be orally administered and the other injected locally.

Particularly preferred embodiment agents are those which are effective against *Janthinobacterium* and/or *Pseudomonas* or *Psuedomonas*-like bacteria.

During the work which led to the present invention, the inventors showed that nitric oxide synthase (which appeared to be bacterial in origin) was differentially expressed in tissues with OA. Nitric oxide synthase has

been found to be inhibited even at the transcriptional level by tetracycline compounds and thus such compounds are a preferred class of antibiotics for use in the invention.

The antibacterial agents for use according to the present invention have a sufficiently inhibitory effect on the bacteria within the joint that they cause a measurable and significant improvement in osteoarthritis and its associated symptoms. It is not expected that in all cases treatment will be totally successful but "treatment" according to the present invention should include improvement in one or more of the following areas: pain in and around the joint at rest or on movement, inflammation around the joint, movement of the joint and decay of the cartilage in the joint. Treatment will preferably see an improvement in one or more of these areas but may include prevention or slowing in the further decline of the cartilage, joint movement etc. The nature of OA means that if the development of the disease is arrested, this could be of significant benefit to the patient. If it is intended to introduce engineered tissue or new cells into the site of damage in the joint, then the site is preferably first treated with an antibacterial agent in accordance with the teaching of the invention.

All patients with OA, whether newly diagnosed or at a more advanced stage, can be considered for treatment in accordance with the present invention. Thus, in a further aspect the present invention provides a method of treating a bacterial infection responsible for osteoarthritis in a mammal, which method comprises administering an amount of an antibacterial agent to said mammal which is sufficient to improve one or more of the symptoms of osteoarthritis. Symptoms in which improvement may be observed are discussed above.

Alternatively viewed, according to a method of the invention, a pharmaceutically effective amount of an

antibacterial agent is administered to a patient in need thereof in order to treat osteoarthritis.

A 'pharmaceutically effective' amount can be determined with reference to the various areas discussed herein in which treatment may provide measurable improvements, and selected with reference to the Examples.

Generally, patients in need of such a treatment will be diagnosed as suffering from OA by reference to the clinical definitions provided herein or other medically accepted criteria.

Alternatively viewed, the invention provides a method of improving joint mobility and/or reducing pain and/or inflammation of a joint in a mammal which method comprises administering a pharmaceutically effective amount of an antibacterial agent to said mammal.

Likewise, the invention provides the use of an antibacterial agent in the production of a medicament for improving joint mobility and/or reducing pain and/or inflammation of a joint.

An improvement in joint mobility may be assessed by the patient themselves or their medical adviser. Likewise with pain and inflammation.

The present invention also provides a pharmaceutical composition for use in the treatment of OA, more particularly a bacterial infection responsible for OA, said composition comprising an antibacterial agent together with at least one pharmaceutically acceptable carrier, diluent or excipient. The active ingredient in such compositions may comprise from 0.05% to 99% by weight of the formulation, more preferably 0.1% to 5.0%.

By "pharmaceutically acceptable" is meant that the ingredients must be compatible with other ingredients of the composition as well as physiologically acceptable to the recipient.

The pharmaceutical compositions may be formulated

according to any of the conventional methods known in the art and widely described in the literature. Thus, the active ingredient may be incorporated, optionally together with other active substances, with one or more conventional carriers, diluents and/or excipients, to produce conventional galenic preparations such as tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments, soft and hard gelatin capsules, suppositories, sterile injectable solutions sterile packaged powders, and the like. Other methods of formulating the antibacterial agents, e.g. by incorporation into devices for implantation, are discussed above.

The active agents are preferably formulated into tablets, each tablet containing a predetermined amount of active ingredient. As discussed herein, it may be desirable to inject certain antibiotics into the affected joint.

Suitable doses will vary from patient to patient and can be determined by the physician in accordance with the weight, age and sex of the patient and the severity of the condition and also the particular antibacterial agent selected. A typical total daily dose of antibacterial agent will be in the region of 50-1000 mg, preferably 100-300 mg. This will preferably be administered as a single dose.

Improvements in patients treated in accordance with the present invention may be seen within a week or two and treatment should normally be continued for 1 to 2 months or more to achieve maximum benefits. As shown herein, 4 weeks may be sufficient to see significant improvements.

The identification of a role for bacteria in the development of OA also provides new methods for the accurate diagnosis of OA. Biopsies taken from patients suspected of having OA, e.g. samples of synovial fluid,

can be tested for the presence of pathogenic bacteria. Thus, in a further aspect, the present invention provides a method of diagnosing osteoarthritis in a patient, which method comprises testing a sample from a joint of said patient for the presence of (pathogenic) bacteria associated with osteoarthritis. Such a method is preferably *in vitro*, practised on a sample taken from the patient which is not returned.

A particularly preferred method involves the use of nucleic acid probes or primers designed to detect the bacterial species of interest through homology with a target region (sequence) within the bacteria's nucleic acid. Suitable primers are described herein in the Examples. Preferably the primer or primers target a region within the 16S rRNA (or the gene encoding it). Primers designated herein as F21- and R21-, particularly F21-4 and R21-4 are specific for *Janthinobacterium* type sequences and are especially preferred. The primer pair F21-2 and R21-4 are also particularly suitable.

The probes/primers have homology with target sequences, i.e. they are capable of binding to target sequences under standard levels of stringency.

Methods for obtaining suitable samples from patients suspected of having OA in one or more joints are provided in the Examples herein. The diagnostic method described herein provides a useful and reliable test for confirming that a joint is affected by OA and can be used on its own or together with known diagnostic techniques.

The sample tested will preferably be synovial fluid. If only a little fluid is obtained then saline solution (possibly up to about 30ml) can be injected before further aspiration. These fluid samples can then be spun down to yield a cell sample, e.g. by centrifuging at 13000-rpm (15000g) for 45 mins. The cells in the pellet are preferably washed with a sterile saline solution 2-3 times before being frozen, e.g. at -

70°C.

The assay for the bacterial gene marker may be based on identification of RNA (e.g. directly for 16S rRNA) or DNA (e.g. the gene encoding 16S rRNA). Where RNA is analysed, a reverse transcription step is required to generate cDNA before PCR can be performed. Methods of analysing RNA are described in the Examples but the DNA from bacteria in the synovial fluid may be analysed instead/as well. The DNA may be first isolated from the cells, e.g. using QIAGEN's DNeasy tissue kit (Cat.No. 69504). Alternatively, the DNA may be amplified and analysed without a separate extraction step. Suitable protocols for amplification of DNA in blood cells is described by Nordvåg, B. et al. in Methods in Neurosciences Vol.26 [1995] p.15-25 and in BioTechniques [1992] Vol.12 No.4, p.490-491 and the methods apply equally to a cell sample from synovial fluid.

When performing PCR, preferably three separate primer pairs will be used:

A) a primer set specific for the disease causing bacteria, so specific for *Janthinobacteria* and related species (preferably designed for amplification of the 16S rRNA gene)

B) a primer set for amplification of another gene in the disease causing bacteria which is not as highly conserved as the 16S rRNA genes, e.g for part of one of the 23S rRNA genes; and

C) a human standard gene such as β-actin which can be used for normalization of the amount of bacterial signal to be a signal of human genes derived from human synovial cells.

The assay may quickly and conveniently be performed using only primer set (A) above.

Cells will preferably lyse in a pre-cycle to the PCR method to make the DNA accessible for primer annealing. After PCR, standard gel visualisation

techniques are used.

Alternatively viewed, the present invention provides the use of a bacterial detection moiety in the manufacture of an agent for the diagnosis of osteoarthritis. As discussed above suitable bacterial detection moieties include nucleic acid probes and primers which may be designed to detect bacteria generally or a particular genus or species. In particular moieties which can detect the *Janthinobacterium* related species which has been found by the present inventors to be linked to OA. Other moieties for bacterial detection include antibodies.

The 'agent' may simply be a solution, suspension etc. which contains the bacterial detection moiety and is or is capable of being in a form convenient for performing the diagnostic method on a sample. The agent will typically be contacted with the sample in order to determine whether or not bacteria are present.

In a further aspect, the present invention provides a kit for the diagnosis of OA which comprises a bacterial detection moiety, preferably an agent as defined above. The 'bacterial detection moiety' is typically one or more oligonucleotide molecules, e.g. a pair of nucleic acid primers which detect the target bacteria responsible for the OA symptoms. The kit may preferably also include one or more of the following a DNA polymerase (which is as free from contaminating DNA as possible), dNTPs, buffers and a reagent to aid RNA/DNA solubilisation.

In a still further aspect, the present invention provides a product containing (a) an antibacterial agent as defined herein and (b) a nitric oxide antagonist as a combined preparation for simultaneous, separate or sequential use in the treatment of OA, typically the treatment of a bacterial infection which is responsible for OA. A 'nitric oxide antagonist' is any moiety which serves to lower the local nitric oxide concentration in

the area surrounding the joint, for example an inhibitor of nitric oxide synthase.

In a further aspect, the present invention provides a product containing (a) an antibacterial agent as defined herein and (b) an agent which can break down DNA, this agent will preferably be an enzyme such as DNaseI, as a combined preparation for simultaneous, separate or sequential use in the treatment of OA, typically the treatment of a bacterial infection which is responsible for OA.

The invention will be further described with reference to the following non-limiting Examples and the Figures in which:

Figure 1 is a sketch showing notchplasty during reconstruction of the anterior cruciate ligament. Cartilage is removed from the lateral wall of the notch.

Figure 2 is a photograph based on optical visualisation showing focally damaged cartilage on the medial femoral condyle. The picture was taken during arthroscopy.

Figure 3 is a photograph based on optical visualisation showing advanced osteoarthritic changes in a knee, again the picture was taken during arthroscopy.

Figure 4 a flow chart of SMART technology. The figure is adapted from the Clontech SMART™ PCR cDNA Synthesis Kit user manual.

Figure 5 is a gel photograph showing the results of re-amplification of differentially expressed bands. The bands between 200-600bp are isolated and used for cloning, sequencing and further verification.

Figure 6 is the first sequence isolated from patients by differential display. It was a FASTA analysis of this sequence which first lead the inventor to suspect the presence of bacteria.

Figure 7 is a gel photograph showing detection of 16S ribosomal RNA signals from control cultures of *E. coli*. Lane 1 = control bacterial RNA, Lane 2 = 1/10 x bacterial RNA, Lane 3 = Water. Reverse transcription is performed with primer R1492 and PCR with F27/R1492; 5 µl of each PCR reaction was applied to the gel.

Figures 8 is a gel photo showing the 16S signal in cartilage biopsies. The lanes were loaded as follows:

Lane	Sample ID
1	8A
2	8A
3	17A
4	17A
5	20A
6	20A
7	16N
8	16N
9	Treated master mix, + primers but no template.

Reverse transcription was performed with primer R1474, PCR with primers F7/R1474 and 6 µl of each PCR reaction was applied to the gel.

Figure 9 is a gel photo showing the 16S signal in cartilage biopsies. The lanes were loaded as follows:

Lane	Sample ID
1	8A
2	17A
3	16N
4	Water control

Reverse transcription and PCR were performed using the primers of Fig. 8.

Figure 10 is a photograph showing purified amplified 16S DNA fragments prior to sequencing. In some samples, especially in normal tissues, a smaller fragment (350bp) relative to that of the 16S signals (1400bp), was observed. These smaller fragments when sequenced appeared to be similar to 18S human rRNA. The gel was loaded as follows:

Lane	Sample ID
1	17A small fragment*
2	17A large fragment
3	20A large fragment
4	17A small fragment*
5	17A large fragment
6	Treated master mix, + primers (F7 and R1474) but no template.

4 μl of each DNA isolate was applied to the gel.

Figure 11 is a diagramatic representation showing primer location in full length 16s rRNA. The F7/R1474 pair will amplify the full-length 16s rRNA cDNA but when used for sequencing, these primers will give sequence information for some few hundred basé-pairs each, which will not cover the complete sequence. To get the complete sequence of the amplified cDNA, the primers F10A, F10B. F10C, R10A, R10B and R10C are used as additional sequencing primers. The nucleotide sequences are listed in Table 1. In this way, a composite sequence using overlapping sequence information obtained by sequencing with several forward primers is generated. The same process may be done with the reverse primers to obtain a composite reverse sequence and then the forward and reverse sequences compared to make an even more

precise composite sequence.

Figure 12 is the full length 16s RNA sequence obtained as a forward and reverse composite sequence from patient number 21, A region, using the primers described in the legend to Figure 16. This sequence was always found in osteoarthritic patents and was found by a Blastn search of the NCBI database to represent *Janthinobacterium*.

Figure 13 is a schematic representation of how different primers may be used to differentiate between *Janthinobacterium* (J) and *Burkholderia* (B) 16s rRNA sequences. These sequences are generally rather similar but with regions where there is a high percentage of mismatch between the two species. Thus, F25-/R25- primers will amplify templates with a B-type sequence and F21-/R21- primers will amplify templates with a J-type sequence. This technique helped in reaching the conclusion that the bacterial species involved in OA was *Janthinobacterium* or very closely related thereto and in identifying the best primers for use in diagnosis of OA caused by bacterial infection, F21-1 and R21-4.

Figure 14 is a gel photograph showing detection of pathogen 16s rRNA genes in synovial fluid. DNA was isolated from synovial fluid from an arthritic knee using the TRIzol kit from Gibco BRL/Life Techologies. The 16s rRNA sequence was amplified using primers designed from the 16s rRNA sequence found in sample 21A. The gel was loaded as follows:

Lane	Sample	Primers
1	DNA	F21-1/R21-4
2	DNA 1/10 dilution	F21-1/R21-4
3	Water control	F21-1/R21-4
4	DNA	F21-1/R21-5
5	DNA 1/10 dilution	F21-1/R21-5

6 Water control F21-1/R21-5

L 1 kb Plus DNA ladder, Gibco BRL/Life Techologies

Figure 15 is a gel photograph showing a failure to detect bacterial 16S rRNA in samples from patient 31, normal (N) region and in a water control. The lanes were loaded as follows:

Lane #	Sample	Forw.Prim.	Rev.Prim.
L	1 kb+ ladder		
1	31N-SF DNA 1/10 dil.	F25-1	R25-3
2	31N-SF DNA 1/10 dil.	F25-1	R25-4
3	31N-SF DNA 1/10 dil.	F25-1	R25-5
4	31N-SF DNA 1/10 dil.	F25-2	R25-3
5	31N-SF DNA 1/10 dil.	F25-2	R25-4
6	31N-SF DNA 1/10 dil.	F25-2	R25-5
7	31N-SF DNA 1/10 dil.	F25-3	R25-4
8	31N-SF DNA 1/10 dil.	F25-3	R25-5
9	Water Ctrl.	F25-1	R25-3
10	Water Ctrl.	F25-1	R25-4
11	Water Ctrl.	F25-1	R25-5
12	Water Ctrl.	F25-2	R25-3
13	Water Ctrl.	F25-2	R25-4
14	Water Ctrl.	F25-2	R25-5
15	Water Ctrl.	F25-3	R25-4
16	Water Ctrl.	F25-3	R25-5

If bacteria had been present in any of the samples (even species other than *Burkholderia*) then a signal should have been generated using these forward and reverse primers.

Figure 16 is a graph showing the KOOS scores for 'symptoms' before and after treatment with an antibiotic. $p = 0.100$

Figure 17 is a graph showing the KOOS scores for 'daily activity' before and after treatment with an antibiotic. p = 0.429.

Figure 18 is a graph showing the KOOS scores for 'Sport and Recreation' before and after treatment with an antibiotic. p = 0.008.

Figure 19 is a graph showing the KOOS scores for 'Quality of Life' before and after treatment with an antibiotic. p = 0.182.

Figure 20 is a graph showing the KOOS scores for 'Pain' before and after treatment with an antibiotic. p = 0.386.

Figure 21 is a graph showing the Lysholm single scores before and after treatment with an antibiotic. p = 0.003.

Examples

Example 1 - Identification of bacteria associated with osteoarthritic damage

Clinical Cartilage Biopsies

The ethical committee at Tromsø University Hospital approved the removal and examination of cartilage for this study. Patients undergoing the biopsy procedure, were informed orally and written about the project, and signed a document telling that they accepted the use of the material for this research project.

The three different qualities of cartilage tissues, normal, focally damaged and osteoarthritic, were taken from patients in the following way:

1. The normal cartilage (N) was taken from knees undergoing anterior cruciate ligament reconstruction. To repair the ligament, a graft including a piece of bone from patella, a part of the patellar ligament, and an amount of bone from the proximal tibia were used. When placing the graft into the knee, the procedure includes the removal of some cartilage from the lateral femoral condyle of the knee (Fig 1). This is done to allow sufficient space for the new ligament in flexion and extension of the knee. None of the knees which were used for harvesting normal cartilage had a history of trauma for the last two months, and there were no signs of actual inflammation in these knees. The removed cartilage was used as the normal tissue sample. During operation it was taken out under sterile conditions, and immediately immersed frozen in

liquid nitrogen. Samples were later stored at -75C.

2. The focally damaged cartilage (D) was from areas in knees removed during repair with chondrocyte transplantation (Fig 2), a technique described by Mats Brittberg and coworkers in 1994 (Brittberg M., Landahl A., Nilsson A., Ohlsson C., Isaksson O., Peterson L., N. Engl. J. Med. 1994, Oct 6; 331(14): 889-95). This technique includes taking a biopsy from the knee which is to be repaired from a not weight bearing area of the knee, and from this biopsy the cells are isolated and allowed to multiply in vitro for about three weeks. When a sufficient number of cells is reached, the cells are transplanted into the knee after removal of the damaged cartilage area. The removed and damaged cartilage was used as a sample of damaged cartilage. Also this biopsy was removed under sterile conditions.

3. The osteoarthritic cartilage (A) (Fig. 3) was removed during operation from patients with advanced osteoarthritis to a degree excluding the cell transplantation technique. These knees underwent joint replacement surgery, using prosthesis. During the operative procedure, the cartilage has to be removed, and areas of cartilage with the most advanced changes were taken, harvested under sterile conditions.

Details of other materials used in the experiments

Primers

The following primers were used. R(reverse) primers R-1492 and R-1474 were used for reverse transcription. For PCR, generally the F-27/R-1492 and the F-7/R-1474 pairs were used (F = forward). The sequence of the primers is indicated in Table 1 below.

The primers were bought from Sigma-Genosys. For cDNA synthesis the primers were diluted to 2,5 pmol/μl in water. For PCR the primers were diluted to 50 pmol/μl in water.

F-7/R-1474 are preferred for identification of bacterial 16S rRNA (or the gene encoding it) generally as they anneal to a sequence conserved among several studied bacterial species. F21-2/R21-4 are suitable for identification of the key causal bacterium *Janthinobacterium* (or closely related species).

Primer	Primer Sequence
F2521	5'- GCA AGT CGA ACG GCA GCA CGG GT -3'
F25-1	5'- GGA TAG CCC GGC GAA AGC CGG AT -3'
F25-2	5'- CCT TCG GGC CTC GCG CTA TAG GGT T -3'
F25-3	5'- TCC TTG GCC CTA ATA CGG TCG GGG G -3'
R25-3	5'- CCC CCG ACC GTA TTA GGG CCA AGG A -3'
R25-4	5'- TCC ACC TCT CAG CGG AAT TCC GA -3'
R25-5	5'- GCA ACC CTC TGT TCC GAC CAT TGT -3'
R2521	5'- GAT TAG CTC CCC CTC GCG GGT TGG -3'
F21-1	5'- GGG ATA ACG TAG CGA AAG TTA CGC TA -3'
F21-2	5'- TCG CAA GAC CTC ATG CTC GTG GAG C -3'
F21-3	5'- CGG TGA GAG CTA ATA TCT CTT GCT AAT -3'
R21-3	5'- ATT AGC AAG AGA TAT TAG CTC TCA CCG -3'
R21-4	5'- CCC TGA TCT CTC AAG GAT TCC AGC C -3'
R21-5	5'- GCG GCG CTC TGT ATG TAC CAT TGT ATC -3'
F-7	5'- ATC CTG GCT CAG ATT GAA CG -3'
R-1474	5'- TCA CCC CAG TCA TGA ATC CT -3'
F-27	5'- AGA GTT TGA TC(C/A) TGG CTC AG -3'
R-1492	5'- TAC GG(C/T) TAC CTT GTT ACG ACT T -3'
F10A	5'- GTG AGT GAA GAA GGC CTT CG -3'
F10B	5'- TGG GGG ATT CAT TTC CTT AF -3'
F10C	5'- AGC AGC CGC GGT AAT ACG -3'
R10A	5'- ATG ACG TGT GAA GCC CTA CC -3'
R10B	5'- TTA ATC CAC ATC ATC CAC CG -3'
R10C	5'- AGC CCG GGG ATT TCA CAT -3'
F27'	5'- AGA GTT TGA TC(C/A) TGG GTC AG -3'
F-8	5'- AGA GTT TGA TCC TGG YTC AG -3'
R-556	5'- CTT TAC GCC CAR TAA WTC CG -3'

Table 1

cDNA synthesis

The Superscript II RNase H⁻ Reverse Transcriptase(Cat.No. 18064-014) from GibcoBRL/Life Technologies were used with its buffer and DTT. dNTP's (Cat.No. U1240) were purchased from Promega.

Agarose gel electrophoresis and DNA isolation

SeaKem LE Agarose(Cat.No. 50004) from MedProbe were used for gel electrophoresis.

QIAEX II Gel Extraction Kit (Cat.No. 20021) from QIAGEN were used for DNA isolation from agarose.

Sequencing kit and conditions

Thermo Sequenase Cy-5 Dye Terminator Kit(Cat.No. 27-2682-01) from Amersham Pharmacia Biotech were used for the sequencing reactions. Sephadex G-50 (Cat.No. 9048-71-9) from Sigma were used for the Sequencing PCR product purification. The ABI PRISM BigDye Terminator Cycle Sequencing Kit could also be used.

PCR reagents

HotStarTaq DNA polymerase (Cat.No. 203205) from Qiagen were used for PCR amplification of 16S ribosomal RNA.

PCR master mixes were treated with RQ1 RNase free DNase I (Cat.No. M610A) from Promega.

Water

RNase free, DNase free, DEPC treated, autoclaved, 0,2 μM filtered water (Cat.No. 9915G) from Ambion were used when preparing RNA, synthesizing cDNA and during PCR.

Differential Display Method

The following technique combines amplification of complete length cDNA with differential display.

SMART cDNA PCR (Clontech Laboratories Inc., PR304-1) is a technique originally developed to amplify complete cDNA populations/libraries. First strand cDNA is synthesized utilizing a oligo-dT primer (CDS primer). The reverse transcriptase (MMLV, RNase H-) adds a small number of cytidine residues after completing the first strand. A second primer (SMART II primer) anneals to the dC-"tail" and the reverse transcriptase switches template. The primers are designed to give the same sequence at both ends of the cDNA and thus by using a primer that will bind to both ends (PCR primer) we can amplify the total mRNA (as cDNA) population exponentially.

We have used SMART cDNA PCR to amplify cDNA constructed from very small mRNA samples. We have then used the amplified cDNA as template in differential display reactions.

Materials and Methods

Sample material were taken from a patient being operated for unicompartment arthrosis in his knee, see Example 1. Biopsies were taken from the osteoarthritic part of the cartilage. From the same knee normal cartilage was taken from the undisturbed area. The cartilage samples were frozen in liquid Nitrogen within 5 minutes and then stored at -75°C until RNA extraction was performed.

RNA extraction

The cartilage were homogenized to a fine powder with a mortar and pestle in liquid Nitrogen. Total RNA was isolated using TRIZOL reagent from Gibco (#15596). RNA concentration and quality were determined by measuring Abs 260/280 and by running a sample on an agarose gel.

cDNA synthesis

Reverse Transcriptions were done using the SMART PCR

cDNA Synthesis Kit from Clontech (#K1052-1). The manufacturer's recommended conditions were followed. 3 μl of RNA solution were used. MMLV reverse transcriptase (200 U/μl)(Gibco 18064-014) were used.

cDNA amplification

Total cDNA was amplified using the SMART PCR cDNA Synthesis Kit (# K1052-1) from Clontech. The manufacturer's recommended conditions were used. 2 μl first-strand cDNA were amplified in a total volume of 50 μl with Advantage 2 KlenTaq Polymerase from Clontech (# 8430-1). After 25 cycles the PCR product were examined on an agarose gel and DNA concentration were determined by measuring Abs 260/280.

Differential Display; using two primers:

For the Differential Display reactions the Delta Differential Display Kit (#K1810-1) from Clontech was used. The manufacturer's recommended conditions were used. The amplified cDNA was diluted to 0.02 μg/μl. Several primer combinations (T and P primers included in the kit) were used to screen for differentially expressed genes.

1 μl of the cDNA dilutions was used as template in each reaction. The samples were labelled with [α]-^{33}P dATP (Amersham) and amplified with Advantage 2 KlenTaq Polymerase (# 8417-1) from Clontech. Following three low-stringency cycles 25 high-stringency cycles were run. The PCR products were separated on a sequencing gel (7 M Urea, 4% acrylamide (37,5:1) 0.5xTBE) with 0,2 mm spacers at 500V until the bromophenol blue dye had left the gel. The gels were visualized by exposing them to BioMax MR film for ½-1 days.

Differentially expressed bands were excised from the

dried gels by superimposing the film to mark the bands prior to isolate the gel material using a scalpel. 100µl water was added to each gel slice in an eppendorf tube. The slices were then incubated at 37°C overnight. The tubes were spun down at 14000g for 15 minutes at 4°C. The eluate were then stored at -20°C. 5 µl of each eluate was used as template in an amplification reaction using the same conditions as in the Differential Display PCR (no labelling). The PCR products were examined on an agarose gel. Some smear was evident and a larger volume was separated on a larger gel. The bands of interest were cut from the agarose gel and the DNA was isolated using the QIAEX II Kit from Qiagen (# 20021). The manufacturer's procedure was followed. DNA was eluted in 20 µl of water. The DNA was reamplified again using *pfu* Turbo polymerase (# 600252-51). Each 50µl reaction contained 1 µl template, 40pM primer T and P, 100µM dNTP, 10xbuffer and 1,25 U of enzyme. PCR was run with the following conditions: Initial 94°C 9 min, 43 cycles of 94°C for 30 sec and 60°C for 1 min and a final elongation at 60°C for 10 min.

After examining a test run on an agarose gel parallel reactions on 2 tubes were run and the products pooled. The DNA was isolated from the PCR reaction mix using the QIAEX II DNA Purification System from Qiagen. The manufacturer recommendations were followed. The DNA was eluted in 15 µl of water. A sample was run on an agarose gel. Upon visualization there were no additional bands or smear visible.

Cloning

The reamplified cDNA was cloned into pGEM-T easy cloning vector (#A1360, Promega, Madison, WI, USA). The ligation was performed with a vector concentration of 50ng/µl. The presence of a plasmid insert of the expected size was assayed by PCR using the same primers

as in the differential display and by cutting the vector with the restriction enzyme *Eco*RI.

The cloned PCR fragments were sequenced using the T7 primer. The sequences found showed strong homology with known human gene sequences. They were also 3-4 times longer than the average EST.

Differential Display using one primer:

Random 10-mer primers (Operon Technologies, Alameda, CA, USA) were used to amplify cDNA with no radioactivity. Each 50μl reaction contained 1μl of cDNA, 80 pM of one primer, 200μM of each dNTP, 10xbuffer and 2.5 U of HotStarTaq polymerase (Qiagen). PCR was run with the following conditions: Initial 95°C 15 min, 40 cycles of 94°C for 2 min, 38°C for 2 min and 68°C for 1 min and 25 sec, and a final elongation at 72°C for 10 min. The PCR amplification products were separated on a 1.5% agarose gel (agarose 1000, Gibco) in TAE buffer with 1 mM guanosine, and visualized with ethidium bromide. Agarose 1000 is able to resolve bands separated by 10 bp. Differentially expressed bands were excised, isolated and cloned into pGEM-T easy vector without additionally amplification.

Results

One important aspect of our strategy is to amplify the total cDNA population prior to using it as template in Differential Display PCR reactions using Clontech Smart™ PCR Kit (Fig. 4).

Comparison of Differential Display PCR products from cartilage samples (healthy and osteoarthritic) showed genes expressed in one sample but not in the other.

After Differential Display the cDNA can be eluted from the gel material. The cDNA can then be reamplified

using the same primers (RT PCR) as in the Differential Display procedure (Fig. 5).

Gene Rescue from the library by Reverse PCR

After sequencing, two internal primers with 100% overlap were designed. These internal primers were used to amplify the whole gene using cDNA library in pTriplEx2 vector (Clontech) as template. The cDNA was packaged in λ TriplEx2 which is converted to pTriplEx2 by transduction into *E. coli* BM25.8. Using the pTriplEx2 cDNA library with the decided internal primers, only the plasmid with the gene of interest is amplified. The primers are designed from the sequence of a differentially expressed gene. After transformation into JM109, the differentially expressed gene is sequenced.

Identification of bacterial 16S ribosomal RNA in cartilage samples

RNA extraction

The fresh-frozen cartilage tissues were homogenized to a fine powder with a mortar and pestle on liquid Nitrogen. Total RNA was isolated using TRIZOL reagent from Gibco(#15596). RNA quality was determined by running a sample on an agarose gel electrophoresis.

First strand cDNA synthesis

The following were mixed in an eppendorf tube:

2 μl RT primer (R1474, 5 pmol)
2 μl RNA sample
8 μl H_2O (DEPC treated, RNase-free)

The tube was heated to 90 ^{0}C for 5 min, then cooled slowly at room temperature.

RT-PCR Master-Mix were prepared:
/One reaction

4	µl	1St Strand buffer
2	µl	0,1 M DTT
1	µl	10 mM dNTP's

To each reaction 7 µl RT-MasterMix were added and the tube was then incubated at 42 °C for 2 min.

To each reaction 1 µl Superscript II Reverse Transcriptase was added. The tubes were then incubated at 42 °C for 50 min. The reactions were stopped by heating at 100 °C for 10 min. The tubes were then quickly placed on ice.

80 µl of DEPC treated water were then added to each tube.

PCR amplification
Preparation and DNase treatment of PCR-MasterMix

1 x rxn		
5	µl	10X buffer
2	µl	25 mM Mg^{2+}
1	µl	10 mM dNTP's
37.5	µl	H_2O
2,5	µl	DNase I

The PCR-MasterMix were incubated at 37 °C for 1 hour, then boiled for 5 minutes and placed on ice. The following reagents were added.

1 rxn		
1	µl	PrimerMix (50 pm/µl of forward primer + 50 pm/µl of reverse primer
0.25	µl	HotStarTaq DNA polymerase

For each PCR reaction 1 µl 1'st strand cDNA were mixed with 49 µl of PCR-MasterMix in a 0,2 ml PCR-tube.

The following PCR were run on a MJ Research PTC-200 Peltier Thermal Cycler.

	95 °C	8 min
	12X	
	95 °C	40 s
	70 °C	40 s
		-1 °C/cycle
	72 °C	2 min
	15X	
	95 °C	40 s
	58 °C	40 s
	72 °C	2 min
	20X	
	95 °C	40 s
	56 °C	40 s
	72 °C	2 min
	4 °C	hold

The PCR products were then stored at -20 °C.

Agarose Gel electrophoresis

The PCR products were run side-by-side on an 0,8 % agarose gel. Mini gels(6x10 cm) were run for 50 min at 90 V in 1X TAE buffer. For Mini gels a typical sample was 6 µl PCR product. A 1 kb Plus DNA ladder from Gibco were run parallel to the samples for DNA fragment size determination.

The gels were stained in EtBr solution (0,5 µg/ml water) for 20 minutes. They were then destained in several washes of water for about 40 min.

The gels were visualized on a BioRad Gel Documentation system.

DNA isolation from agarose gel

Four identical PCR reactions were run in parallel, for the preparative isolation of DNA for sequencing. The combined products were then run on a gel with large wells. The same conditions as in the previous visualization were used. After staining the gels were laid on a low intensity UV transilluminator and the bands of interest were quickly excised with sterile scalpels. We have found out that soaking the gel in 1mM guanosine solution (Grundemann and Schomig) prior to UV visualization of the bands eliminated the complications in DNA sequence due the UV damage of the DNA. A typical gel piece weighed about 150-200 mg.

DNA was isolated from the gel fragments using the QIAGEN QIAEX II Gel Extraction Kit (Cat.No. 20021). The manufacturers conditions were followed. The DNA fragments were eluted in 25 μl 10 mM Tris-HCl, pH 8.5.

The quality of the isolated DNA was checked by running a small amount (2μl) on a Mini gel as described before. An example of purified DNA fragments isolated prior to DNA sequencing is shown in Figure 10.

DNA Sequencing

Cy-5 dNTP mixes

For four sequencing reactions four Cy-5 dNTP mixes were made (A,C,G,T).

4 rxns		(MA,MC,MG,MT mixes)
2	μl	1,1 mM dNTP's
1	μl	Cy-5 dNTP(A,C,G or T)
7	μl	H_2O

1 µl 0,55 mM EDTA

Tubes containing Cy-5 dNTP's must at all times be kept on ice in the dark.

A sequencing master mix were made.

1 rxn

8	µl	DNA template
2	µl	primer(forward or reverse)
3.5	µl	rxn buffer
0.9	µl	Thermo Sequenase
12.6	µl	H_2O

In four PCR tubes the following were added.

A:	2	µl	MA
C:	2	µl	MC
G:	2	µl	MG
T:	2	µl	MT

To each of the four tubes 6 µl of sequencing master mix were added.

The following PCR were run:

30X

95 °C	30 s	
57 °C	30 s	
72 °C	80 s	

The sequencing reaction products were purified on Sephadex G-50 spin columns (96 well array). The products were then dried in the dark at 37 °C and then resuspended in sequencing stop solution (8 µl). The samples (4 µl) were then run on an Amersham-Pharmacia ALF sequencer under standard conditions.

Results

Detection of 16 S RNA in cartilage from patients with osteoarthritis

PCR Results

After we have managed to test the protocol by detecting 16S RNA using diluted bacterial cultures of *Escherichia coli* (Figure 7) we tested this method on cartilage clinical samples.

Designation of lanes/tissues are (N) for normal or non-affected regions, (D) for Damaged and (A) for osteoarthritic. A joint from one patient may have regions of obviously advanced OA, regions which look quite normal and areas which are not in the advanced stages of OA but where the cartilage is soft and to a certain degree fibrillated, this last area is defined as damaged.

The results are shown in the Figures and Table 2 below.

The results show that the presence of 16S signals can always be associated with osteoarthritic patients. Although occasionally tissue isolated from osteoarthritic patients did not reveal 16S RNA signals using the standard PCR conditions, no 16S RNA signal was ever detected in tissues from normal patients.

Table 2 below gives a summary of the detection of 16s rRNA sequences in clinical samples. A = Osteoarthritic and N = normal. Two types of control were used, H_2O control (blank) and *E. coli* total RNA as a positive control. Reverse transcription is done with primer R1474. PCR amplification was performed using F7 and R1474 primers. The numbers in the 'sample' column are the patient numbers. Some samples appear more than once

in the table because the sample was analysed on more than one day.

Table 2

Sample	Positive	Negative
8A	*	
17A	*	
20A	*	
16N		*
E.coli ctrl	*	
H_2O		*
8A	*	
17A	*	
H_2O		*
8A	*	
17A	*	
16N		*
H_2O		*
8A		*
17A	*	
H_2O	*	
21A	*	
H_2O		*
21A	*	
H_2O		*
25A	*	
H_2O		*
25A	*	
H_2O		*

	25A	*	
	H_2O	*	
	25A	*	
	H_2O		*
	25A	*	
	H_2O		*
	25A	*	
	21A	*	
	H_2O	*	
	21A	*	
	H_2O	*	
	8A	*	
	17A	*	
	20A	*	
	16N		*
	H_2O	*	
	17A	*	
	20A	*	
	H_2O	*	
	8A	*	
	17A		*
	20A	*	
	15A		*
	H_2O		*
	8A	*	
	15A	*	
	17A	*	
	20A	*	
	16N		*

H_2O	*	
8A	*	
15A	*	
16N		*
H_2O	*	
8A		*
17A	*	
20A	*	
16N		*
H_2O	*	
8A	*	
15A	*	
17A	*	
20A	*	
16N		*
18N		*
H_2O		*

Sequence Analysis

The first differential display sequence isolated from patients (Figure 6) led us to suspect the presence of bacteria.

Summary of a FASTA analysis of this sequence is shown below and this indicates a very high similarity to sequences of prokaryotic origin.

Sequences producing significant alignments:		(bits)	Value
gb|AE000244.1|AE000244	Escherichia coli K-12 MG1655 section...	657	0.0
dbj|D90788.1|D90788	E.coli genomic DNA, Kohara clone #277(3...	657	0.0
dbj|D90787.1|D90787	E.coli genomic DNA, Kohara clone #276(3...	657	0.0
emb|X94992.1|ECNARG	E.coli nitrite extrusion gene and secon...	657	0.0

dbj|D26057.1|STYNARK	Salmonella typhimurium genes for SmvA ...	156	2e-35
gb|AE000220.1|AE000220	Escherichia coli K-12 MG1655 section...	79	2e-12
dbj|D90757.1|D90757	Escherichia coli genomic DNA. (27.3 - 2...	79	2e-12
emb|X69189.1|ECNARXLO	E.coli narXL operon and partial narK ...	79	2e-12
emb|X15996.1|ECNARK	E.coli narK gene and partial sequence o...	79	2e-12
gb|AF026945.1|AF026945	Homo sapiens cig64 mRNA, partial seq...	57	1e-05
gb|U32804.1|U32804	Haemophilus influenzae Rd section 119 of...	43	0.18
gb|AC009276.9|AC009276	Homo sapiens chromosome 7 clone RP11...	41	0.74
gb|AE003592.1|AE003592	Drosophila melanogaster genomic scaf...	41	0.74
gb|AF098951.1|AF098951	Homo sapiens breast cancer resistanc...	41	0.74
gb|AF095856.1|AF095856	Homo sapiens asthmatic clone 4 mRNA,...	41	0.74
gb|AF095855.1|AF095855	Homo sapiens asthmatic clone 3 mRNA,...	41	0.74
gb|AF100329.1|AF100329	Dendrobium grex Madame Thong-IN ovgl...	41	0.74
gb|AF013290.1|AF013290	Meloidogyne incognita elongation fac...	41	0.74
emb|X65318.2|CVPGEMEX2	Cloning vector pGEMEX-2	41	0.74
emb|X65317.2|CVPGEMEX1	Cloning vector pGEMEX-1	41	0.74
gb|L36849.1|SYNSHBL	Cloning vector pZEO (isolate SV1) phleo...	39	3.0
ref|NM_015880.1|	Homo sapiens RIG-like 14-1 (LOC51047), mRNA	39	3.0
gb|L36850.1|SYNLACZ	Cloning vector pZEO (isolate SVLacZ) be...	39	3.0
gb|AE003496.1|AE003496	Drosophila melanogaster genomic scaf...	39	3.0

Composite sequences from various patient samples generated using the primers described herein have been subjected to FASTA analysis. The results of these indicate the presence in OA tissue of *Janthinobacterium* or a bacterial species very closely related thereto. Two such analyses are presented below by way of example.

The forward and reverse composite sequence from affected tissue of patient 21 (Fig. 12) was found by Blastn search of the NCBI data-base to represent *Janthinobacterium*. This sequence was always found in OA patients.

The 3 best alignments of this sequence following a Blastn enquiry of November 13, 2000 were:

gi|3201903|gb|AF067655.1|AF067655	Uncultured Duganella clon...	2730	0.0

gi|5738214|gb|AF174648.1|AF174648 Janthinobacterium lividum... 2714 0.0

gi|2832894|emb|Y08846.1|JL16SRRN J.lividum 16S rRNA gene 2714 0.0

The actual alignments were as follows:

>gi|3201903|gb|AF067655.1|AF067655 Uncultured Duganella clone CTHB-18 16S ribosomal RNA gene, partial
sequence

Length = 1453

Score = 2730 bits (1377), Expect = 0.0

Identities = 1393/1400 (99%)

Strand = Plus / Plus

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Query: 344  gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc 403
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Sbjct: 387  ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa 446

Query: 464  tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta 523
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Sbjct: 1407 taaccgtaaggagggcgctt 1426
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>gi|5738214|gb|AF174648.1|AF174648 Janthinobacterium lividum 16S ribosomal RNA gene, partial sequence

Length = 1486

Score = 2714 bits (1369), Expect = 0.0

Identities = 1391/1400 (99%)

Strand = Plus / Plus

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Sbjct: 1407 taaccgcaaggagggcgctt 1426
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>gi|2832894|emb|YO8846.1|JL16SRRN J.lividum 16S rRNA gene

Length = 1469

Score = 2714 bits (1369), Expect = 0.0

Identities = 1391/1400 (99%)

Strand = Plus / Plus

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Query: 224  ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag 283
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Sbjct: 206  ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag 265

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Sbjct: 446  tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta 505

Query: 524  gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc 583
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 644  ggcagaggggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac 703
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct: 746  aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc 805

Query: 824  ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca 883
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 806  ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca 865

Query: 884  agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat 943
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 866  agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat 925

Query: 944  tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccttgagagatcag 1003
            ||||||||||||||||||||||||||||||||||||||||||||||||  ||||||  |
Sbjct: 926  tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccccgagagattgg 985
```

```
Query: 1004 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt 1063
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 986  ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt 1045

Query: 1064 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg 1123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1046 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg 1105

Query: 1124 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca 1183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1106 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca 1165

Query: 1184 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc 1243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1166 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc 1225

Query: 1244 cgcgaggggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga 1303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1226 cgcgaggggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga 1285

Query: 1304 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcncggtnaanacgttccc 1363
            |||||||||||||||||||||||||||||||||||||||||| |||| || ||||||||
Sbjct: 1286 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc 1345

Query: 1364 gggtcttgtacacaccgcccgtcacaccatgggagcgggttttaccagaagtaggtagct 1423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1346 gggtcttgtacacaccgcccgtcacaccatgggagcgggttttaccagaagtaggtagct 1405

Query: 1424 tanccncaaggagggcgctt 1443
            || || ||||||||||||||
Sbjct: 1406 taaccgcaaggagggcgctt 1425
```

A composite sequence (705 nucleotides) from affected tissue in patient 17 using forward primer F7 was similarly analysed and the four best allignments from a database containing 671,573 sequences were:

gb|AF174648.1|AF174648	Janthinobacterium lividum 16S riboso...	1315	0.0
gb|AF067655.1|AF067655	Uncultured Duganella clone CTHB-18 1...	1315	0.0
dbj|AB021388.1|AB021388	Pseudomonas mephitica DNA for 16S r...	1315	0.0
emb|Y08846.1|JL16SRRN	J.lividum 16S rRNA gene	1315	0.0

The actual alignment for the first sequence on the list is shown below:

gb|AF174648.1|AF174648 Janthinobacterium lividum 16S ribosomal RNA gene, partial sequence

Length = 1486

Score = 1315 bits (658), Expect = 0.0

Identities = 681/689 (98%), Gaps = 3/689 (0%)

Strand = Plus / Plus

```
Query: 19  gagcttgctctggtggcgagtggcgaacgggtgagtaatatatcggaacgtaccctagag 78
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 50  gagcttgctctggtggcgagtggcgaacgggtgagtaatatatcggaacgtaccctagag 109

Query: 79  tgggggataacgtagcgaaagttacgctaataccgcatacgatctaaggatgaaagtggg 138
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 110 tgggggataacgtagcgaaagttacgctaataccgcatacgatctaaggatgaaagtggg 169

Query: 139 ggatcgcaagacctcatgctcgtggagcggccgatatctgattagctagttggtagggta 198
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 170 ggatcgcaagacctcatgctcgtggagcggccgatatctgattagctagttggtagggta 229

Query: 199 aaagcctaccaaggcatcgatcagtagctggtctgagaggacgaccagccacactggaac 258
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 230 aaagcctaccaaggcatcgatcagtagctggtctgagaggacgaccagccacactggaac 289
```

```
Query: 259 tgagacacggtccagactcctacgggaggcagcagtggggaattttggacaatgggcgaa 318
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 290 tgagacacggtccagactcctacgggaggcagcagtggggaattttggacaatgggcgaa 349

Query: 319 a--ctgaatccagcaatgccgcgtgagtgaagaaggccttcgggttgtaaagctcttttg 376
           |  |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 350 agcctga-tccagcaatgccgcgtgagtgaagaaggccttcgggttgtaaagctcttttg 408

Query: 377 tcagggaagaáacggtgagagctaatatctcttgctaatgacggtacctgaagaataagc 436
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 409 tcagggaagaaacggtgagagctaatatctcttgctaatgacggtacctgaagaataagc 468

Query: 437 accggctaactacgtgccagcagccgcggtaatacgtagggtgcaagcgttaatcggaat 496
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 469 accggctaactacgtgccagcagccgcggtaatacgtagggtgcaagcgttaatcggaat 528

Query: 497 tactgggcgtaaagcgtgcgcaggcggttttgtaagtctgatgtgaaatccccgggctca 556
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 529 tactgggcgtaaagcgtgcgcaggcggttttgtaagtctgatgtgaaatccccgggctca 588

Query: 557 acctgggaattgcattggagactgcaaggctagaatctggcagagggggggtagaattcca 616
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 589 acctgggaattgcattggagactgcaaggctagaatctggcagagggggggtagaattcca 648

Query: 617 cgtgtagcagtraaatgcgtagatatgtggaggaacaccgatggcgaagsvagccccctg 676
           |||||||||||| ||||||||||||||||||||||||||||||||||  |||||||||
Sbjct: 649 cgtgtagcagtgaaatgcgtagatatgtggaggaacaccgatggcgaaggcagccccctg 708
```

```
Query: 677 ggtcaagawtgacgctcatgcacaaaagc 705
           |||||||| |||||||||||||| |||||
Sbjct: 709 ggtcaagattgacgctcatgcacgaaagc 737
```

Isolation of RNA and DNA from synovial fluid (SF)

The samples discussed previously were from biopsies but in a significant advance, it has now also been shown that a diagnosis can be made from a sample of synovial fluid which can be obtained without surgery in a much less invasive and traumatic procedure. A suitable protocol is described below.

After collection of SF from the patient the SF was stored at 4°C and after no more than 20 minutes it was centrifuged at 13000 rpm (approx. 12500 g) for 30 minutes at 4°C. The supernatant was removed and the pellet was stored at -72°C until RNA/DNA isolation. 1 ml of TRIzol solution was added to the pellet. The pellet was dissolved by vortexing and then incubated at room temperature for 10 minutes. 0.2 ml of chloroform was added to the solution and it was then mixed by vortexing and then incubated at room temperature for 15 minutes.

The solution was then centrifuged at 13000 rpm for 15 minutes at 4°C. The upper, aqueous phase was removed and RNA was isolated as described before. 0.3 ml of ethanol was added to the phenol-chloroform phase and the solution was then mixed by vortexing. The solution was then incubated at room temperature for 15 minutes. It was then centrifuged at 2000 rpm (approx. 4500) for 5 minutes at 4°C.

The supernatant was removed and the pellet was washed three times in 0.5 ml 0.1 M NaCitrate with 10% ethanol. After each wash it was centrifuged at 2000 rpm (approx. 4500 g) for 5 minutes at 4°C and the supernatant was discarded. After the last wash the pellet was air-dried for 30 minutes at room temperature. The pellet was resuspended in 80 μl of 8 mM NaOH.

Example 2 - Antibiotic treatment of patients with osteoarthritis - Clinical Aspects

In this study an antibiotic effective against a wide range of Gram-negative and Gram-positive bacteria was used.

Introduction:

Thirty patients were diagnosed and treated for one month with the antibiotic known generically internationally as Doxycycline, this was purchased as Vibramycin from Pfizer. Samples from synovial fluid were taken before and after treatment.

Methods:

We identified 30 patients that had clinical and radiological signs of Osteoarthritis. These patients were informed and signed a paper according to the instructions from the ethical committee (Northern Norway Health Region 5).

We used the KOOS (Knee Injury and Osteoarthritis Outcome Score) [Roos et al: Development of a self-administered outcome measure. Journal of Orthopaedic and Sports Physical Therapy 78(2): 88-96, 1998] and the Lysholm score [Ref. Tegner and Lysholm: Rating systems in the Evaluation of Knee Ligament Injuries. Clinical Orthopaedics an Related Research Number 198 Sept. 1985: 43-49.]

During the first visit we took a sample from the synovial fluid. Standard technique, upper lateral portal, using a 10ml. syringe. If there was too little fluid and we did not aspirate synovial fluid at once, we injected 5-10 ml. physiological saline water and again aspirated. The samples were immediately put into an

icebox and brought to the lab. All the patients were given Vibramycin 100mg daily for 4 weeks. From the patients where we found the bacteria, we took new synovial-fluid samples using the same technique.

Clinical results:

We observed improvement.

It is important to note that in this scoring system higher values means that the patients are better. Low values more symptoms, pain e.g.
All values improved in this study. A particularly significant improvement is considered when p values are ≤ 0.05.

KOOS: These parameters were analysed.

Symptoms
Activity of daily living
Sport
Quality of daily living
Pain

Lysholm:

0-100. With 100 is the best score possible and 0 is the worst.

The results of these tests are presented in graphical form in Figs. 16 to 21.

Table 3 below show a summary of the response to antibiotic treatment with tetracycline for four weeks as determined by direct questions to the patients on how they felt.

Sample/Patient	Status after 4 weeks of treatment
2	improved
3	unchanged
4	unchanged
5	unchanged
6	improved
7	improved
8	unchanged
9	improved
10	improved
11	unchanged
12	unchanged
13	improved
14	improved
15	unchanged
16	improved
17	unchanged
18	unchanged
19	unchanged
20	unchanged
21	unchanged
22	unchanged
23	unchanged
24	unchanged
25	unchanged
26	improved
34	improved

Conclusion

These results support our hypothesis that osteoarthritis can be treated with antibiotics. Vibramycin improved all the clinical scores for the patient.

CLAIMS:

1. Use of an antibacterial agent in the manufacture of a medicament for the treatment of osteoarthritis.

2. Use of an antibacterial agent in the manufacture of a medicament for the treatment of a bacterial infection which is responsible for osteoarthritis.

3. Use of an antibacterial agent in the manufacture of a medicament for preventing or reducing the development of advanced stage osteoarthritis.

4. A use as claimed in any preceding claim wherein said antibacterial agent is effective against *Janthinobacterium*.

5. A use as claimed in any preceding claim wherein said antibacterial agent is selected from the group comprising: clariththromycin, levofloxacin, mercaptoethylguanidine, ciprofloxacinlactate, tobramycin, ceftazidimpentathydrate, gentamicin, ciproxin, rifampicin, doxycycline, trimetroprim and sulfamethoxazole.

6. A use as claimed in any preceding claim wherein said medicament comprises two or more antibacterial agents.

7. A use as claimed in any preceding claim wherein the treatment further comprises the administration of a second medicament containing a further antibacterial agent or an agent which can break down DNA.

8. A use as claimed in claim 7 wherein said second medicament contains gentamicin, ciproxin or DNaseI.

9. A use as claimed in claim 8 wherein said second medicament is in a form suitable for injection.

10. A method of diagnosing osteoarthritis in a patient, which method comprises testing a sample from a joint of said patient for the presence of bacteria.

11. A method as claimed in claim 10 wherein the bacteria are detected through binding of one or more oligonucleotides to the nucleic acid of said bacteria.

12. A method as claimed in claim 10 or 11, wherein said sample is contacted with a pair of primers capable of binding to target sequences within the nucleic acid of said bacteria.

13. A method as claimed in claim 12 wherein said primers are capable of binding to regions within the bacteria's 16S rRNA or the gene coding therefore.

14. A method as claimed in any one of claims 10 to 13 wherein said sample is a sample of synovial fluid.

15. A kit for the diagnosis of osteoarthritis which comprises a bacterial detection moiety and optionally a DNA polymerase.

16. A kit as claimed in claim 15 wherein said bacterial detection moiety is a pair of primers as defined in claims 12 or 13.

17. A product containing (a) an antibacterial agent and (b) a nitric oxide antagonist as a combined preparation for simultaneous, separate or sequential use in the treatment of osteoarthritis.

18. A product containing (a) an antibacterial agent and (b) an agent which can break down DNA as a combined preparation for simultaneous, separate or sequential use in the treatment of osteoarthritis.

19. A method of treating a bacterial infection responsible for osteoarthritis in a mammal, which method comprises administering an amount of an antibacterial agent to said mammal which is sufficient to improve one or more of the symptoms of osteoarthritis.

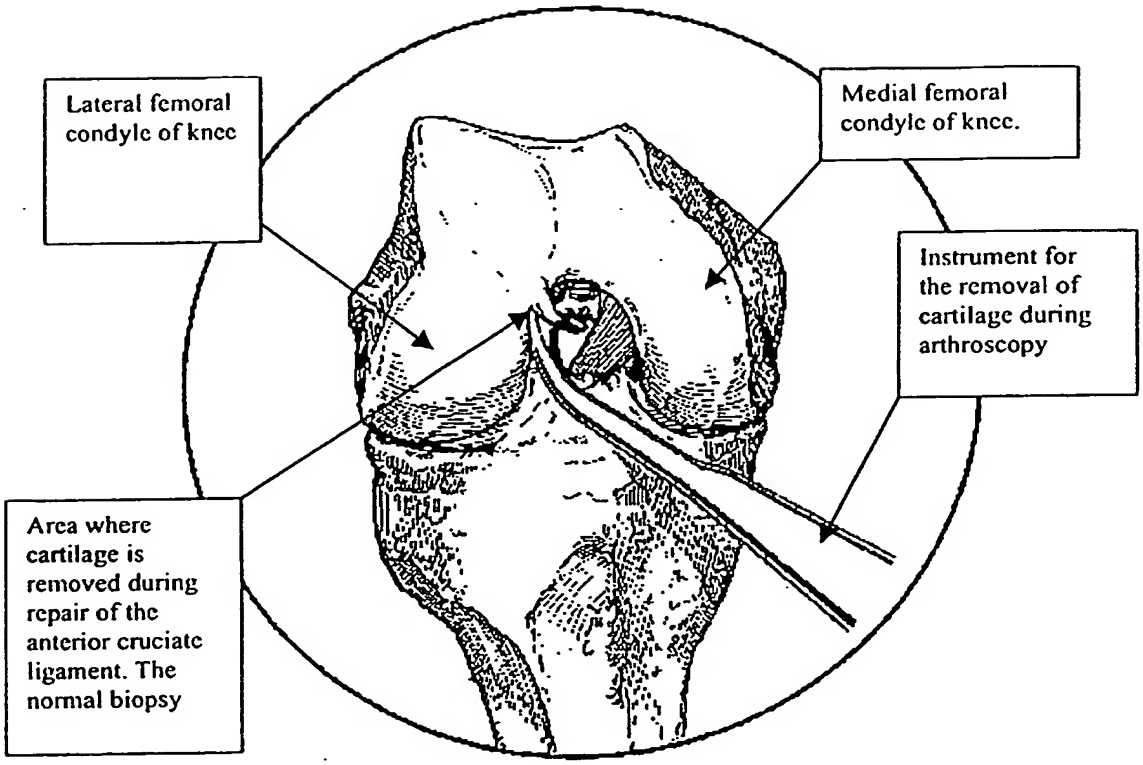
Lateral femoral condyle of knee
Medial femoral condyle of knee.
Instrument for the removal of cartilage during arthroscopy
Area where cartilage is removed during repair of the anterior cruciate ligament. The normal biopsy

Fig 1

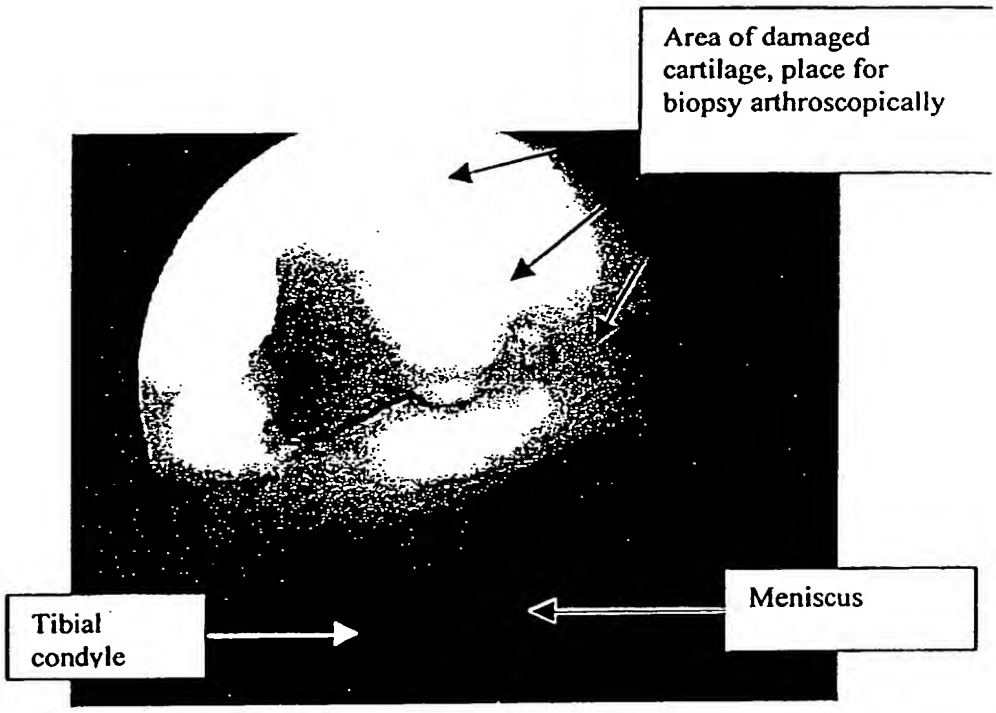
Area of damaged cartilage, place for biopsy arthroscopically
Tibial condyle
Meniscus

Fig. 2

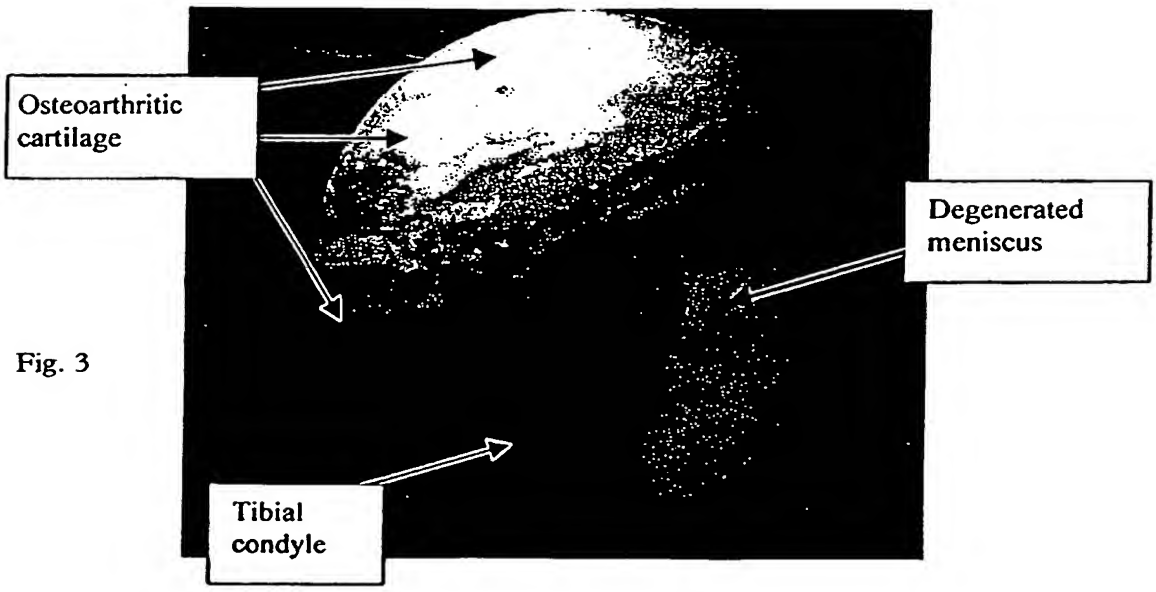
Osteoarthritic cartilage
Degenerated meniscus
Tibial condyle

Fig. 3

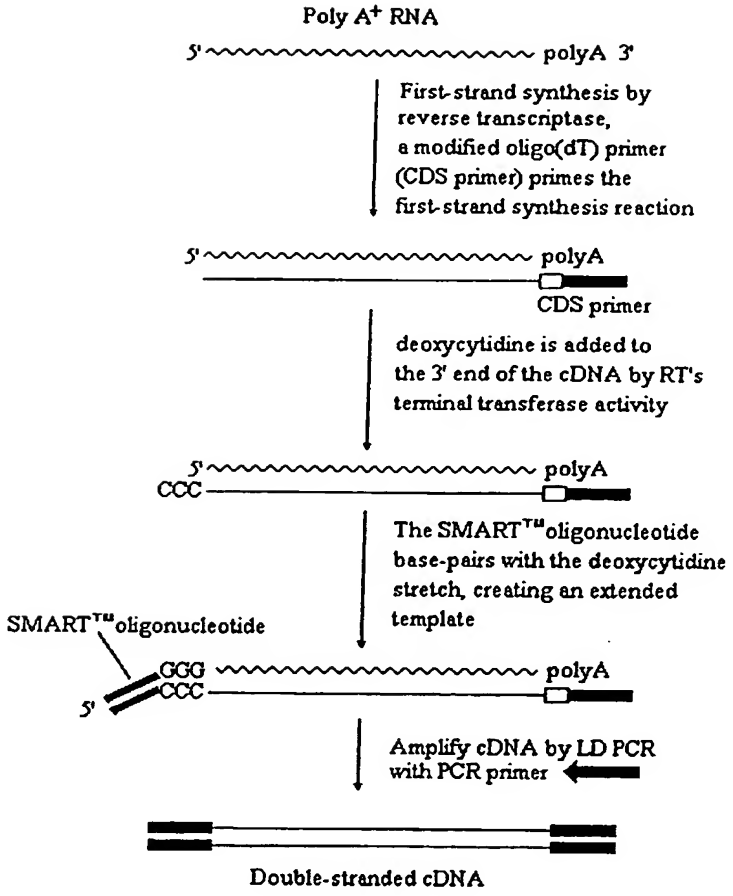
Poly A+ RNA
5'
polyA 3'
First-strand synthesis by reverse transcriptase, a modified oligo(dT) primer (CDS primer) primes the first-strand synthesis reaction
5'
polyA
CDS primer
deoxycytidine is added to the 3' end of the cDNA by RT's terminal transferase activity
5'
polyA
CCC
The SMART™ oligonucleotide base-pairs with the deoxycytidine stretch, creating an extended template
SMART™ oligonucleotide
GGG
CCC
5'
polyA
Amplify cDNA by LD PCR with PCR primer
Double-stranded cDNA

Fig 4

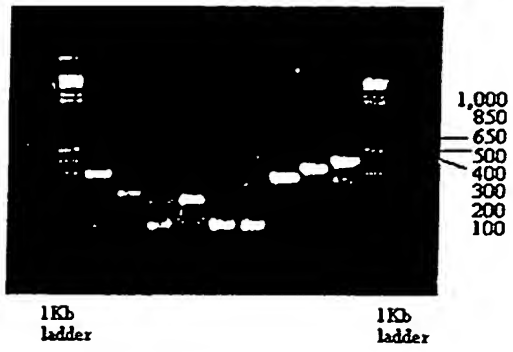
1,000
850
650
500
400
300
200
100
1Kb ladder
1Kb ladder

Fig 5

```
   1  gcgactggaa acCAGAAAAT MCGGCCTTCT GGGARRATAA AGGAARACAT
  51  ATTGCTCGAA GAAATCTCTG GATATCAGTC AGTTGTCTAC TTCTTGCCTT
 101  CTGTGTCTGG ATGCTATTTA GCGCAGTTAC CGTTAATCTC AATAAAATCG
 151  GTTTTAATTT TACTACCGAT CAACTCTTTT TTATTAACCC TCACTAAAGC
 201  ACCGTCCATC GGCGTCCACC GAATATAGGC ACCATAAAGG AGTAGGGAAC
 251  ACGCAATAAT GCGCCAGAAA CGGAGGGTAA TGCGGTTAAT AAAAAGAGTT
 301  GATCGGTAGT AAAATTAAAA CCGATTTTAT TGAGATTAAC GGTAACTGCG
 351  CTAAATAGCA TCCAGACACA GAAGGCAAGA AGTAGACAAC TGACTGATAT
 401  CCAGAGATTT CTTCGAGCAA TATGTTTTCC TTTATTTTCC CAGAAGGCCG
 451  GATTTTCTGG TTTCCAGTCG CGCAAAAGAT AACGACTATT TTTCTCAtTT
 501  TBGCAGTGCC ATATTGTTCC TCACATGCAC ATCATTGGTA ACgaaaaaaa
 551  aagatatcac tcagcataat gagaaaaata gtcgttatct tttgcgcgac
 601  trgaaaccwk aaaatccggy cttctgggaa watamatgga wavcathttg
 651  ctccagaaag tctctggtak cagwctagtb tgmtattcct gashtttctt
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Fig 6

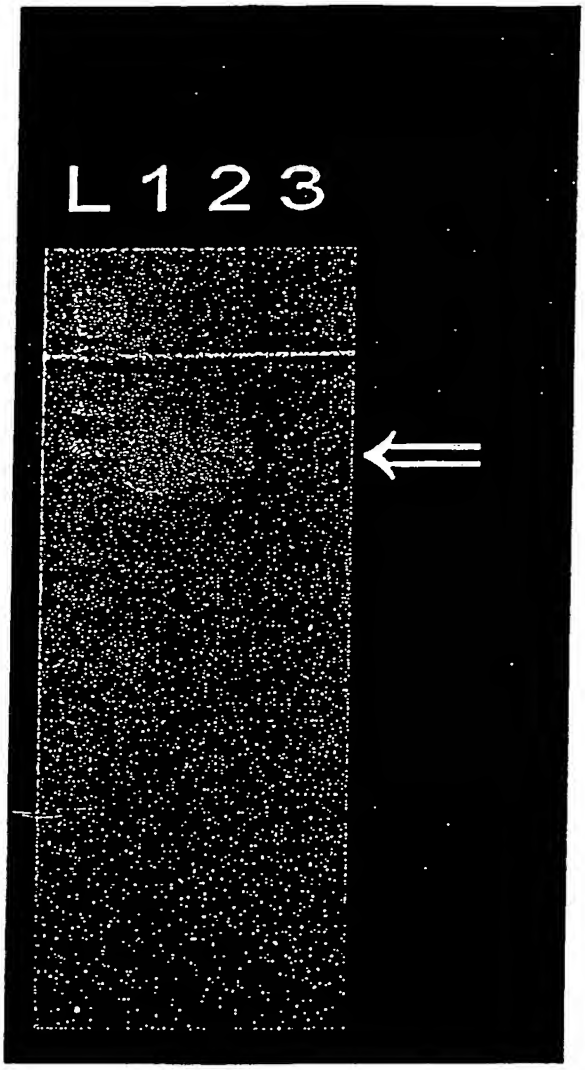


Fig 7

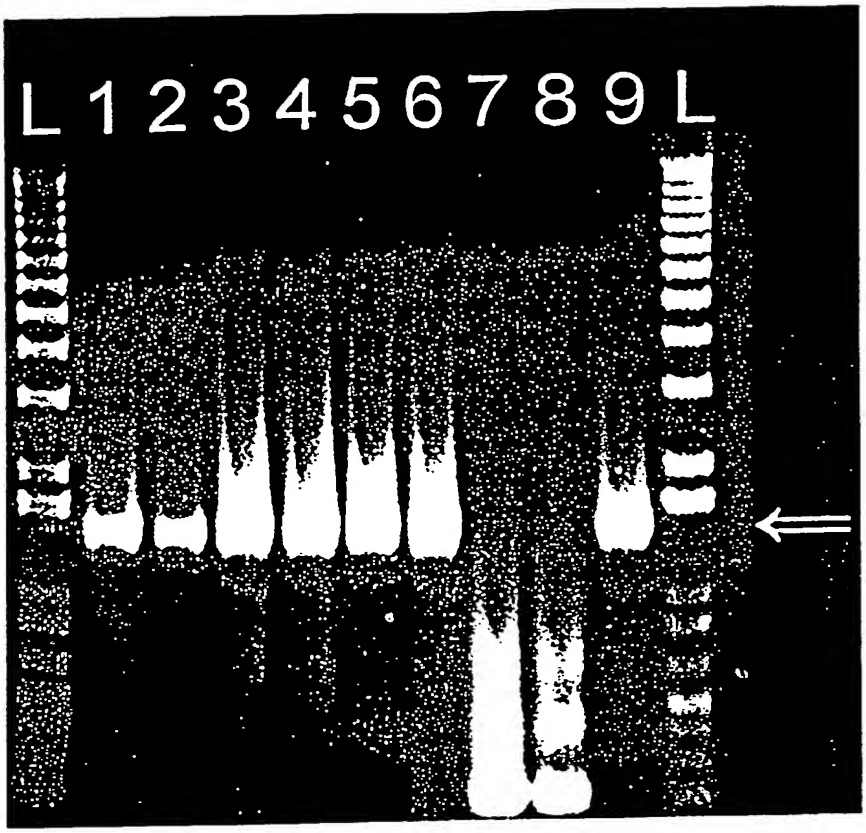


Fig 8

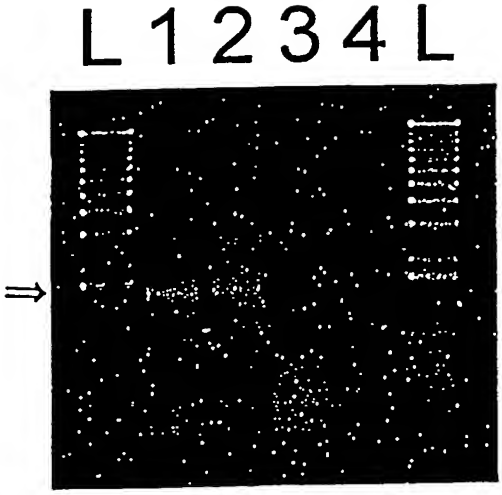


Fig 9

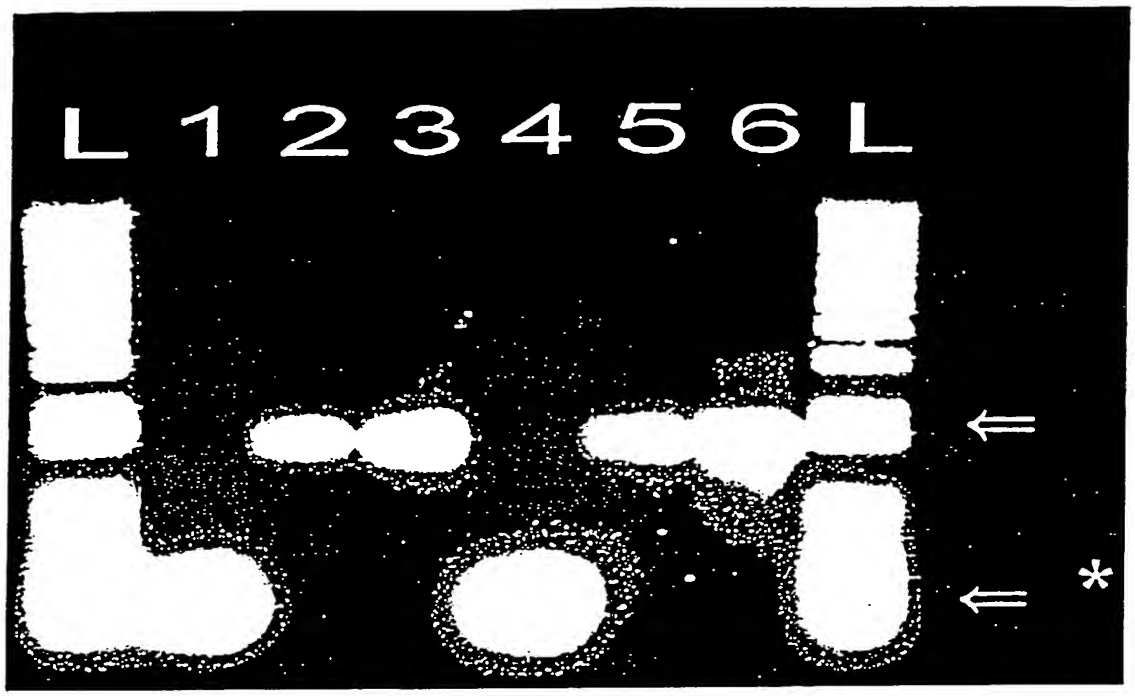


Fig 10

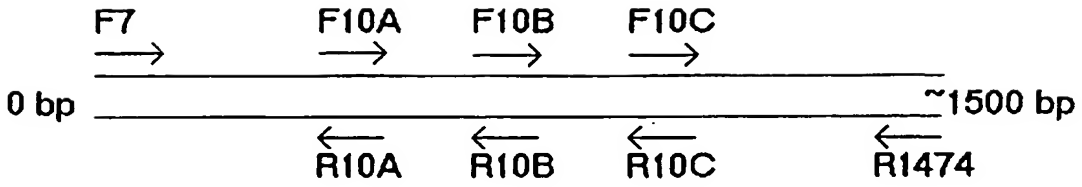


Fig 11

GNGGNNAGTG NNNGNNTNTN NTANTNTNNT NTTGATGCCC CACCATGCAA
GTCGAACGGC AGCACGGAGC TTGCTCTGGT

GGCGAGTGGC GAACGGGTGA GTAATATATC GGAACGTACC CTAGAGTGGG
GGATAACGTA GCGAAAGTTA CGCTAATACC

GCATACGATC TAAGGATGAA AGTGGGGGAT CGCAAGACCT CATGCTCGTG
GAGCGGCCGA TATCTGATTA GCTAGTTGGT

AGGGTAAAAG CCTACCAAGG CATCGATCAG TAGCTGGTCT GAGAGGACGA
CCAGCCACAC TGGAACTGAG ACACGGTCCA

GACTCCTACG GGAGGCAGCA GTGGGGAATT TTGGACAATG GGCGAAAGCC
TGATCCAGCA ATGCCGCGTG AGTGAAGAAG

GCCTTCGGGT TGTAAAGCTC TTTTGTCAGG GAAGAAACGG TGAGAGCTAA
TATCTCTTGC TAATGACGGT ACCTGAAGAA

TAAGCACCGG CTAACTACGT GCCAGCAGCCG CGGTAATAC
GTAGGGTGCA AGCGTTAATC GGAATTACTG GGCGTAAAGC

GTGCGCAGGC GGTTTTGTAA GTCTGATGT GAAATCCCCG
GGCTCAACCT GGGAATTGCA TTGGAGACT GCAAGGCTAG

AATCTGGCAG AGGGGGGTA GAATTCCACG TGTAGCAGTG
AAATGCGTAG ATATGTGGAG GAACACCGAT GGCGAAGGCA

GCCCCCTGGG TCAAGATTGA CGCTCATGCA CGAAAGCGTG GGGAGCAAAC
AGGATTAGAT ACCCTGGTAG TCCACGCCCT

AAACGATGTC TACTAGTTGT CGGGTCTTAA TTGACTTGGT AACGCAGCTA
ACGCGTGAAG TAGACCGCCT GGGGAGTACG

GTCGCAAGAT TAAAACTCAAA GGAATTGACG GGGACCCGCA CAAGCGGTGG
ATGATGTGGA TTAATTCGAT GCAACGCGAA

AAACCTTACC TACCCTTGAC ATGGCTGGAA TCCTTGAGAG ATCAGGGAGT
GCTCGAAAGA GAACCAGTAC ACAGGTGCTG

CATGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC
GAGCGCAACC CTTGTCATTA GTTGCTACGA

AAGGGCACTC TAATGAGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT
GACGTCAAGT CCTCATGGCC CTTATGGGTA

Fig 12

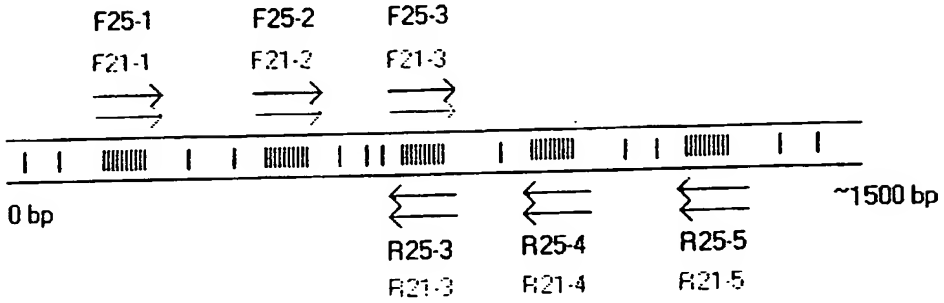
GGGCTTCACA CGTCATACAA TGGTACATAC AGAGCGCCGC CAACCCGCGA
GGGGGAGCTA ATCGCAGAAA GTGTATCGTA

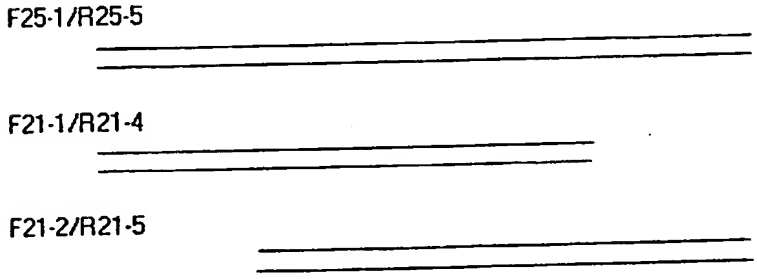
GTCCGGATTG TAGTCTGCAA CTCGACTGCA TGAAGTTGGA ATCGCTAGTA
ATCGCGGATC AGCATGTCNC GGTNAANACG

TTCCCGGGTC TTGTACACAC CGCCCGTCAC ACCATGGGAG CGGGTTTTAC
CAGAAGTAGG TAGCTTANCC NCAAGGAGGG

CGCTTCCAAG GTATNNATCA AANNNNCNNN NNCNNNCCCC
NNNC

Fig 12 cont





DNA Region with many a high percentage of mismatch between B- and J-type sequencel

Single basepair mismatch between B- and J-type sequencel

Fig 13

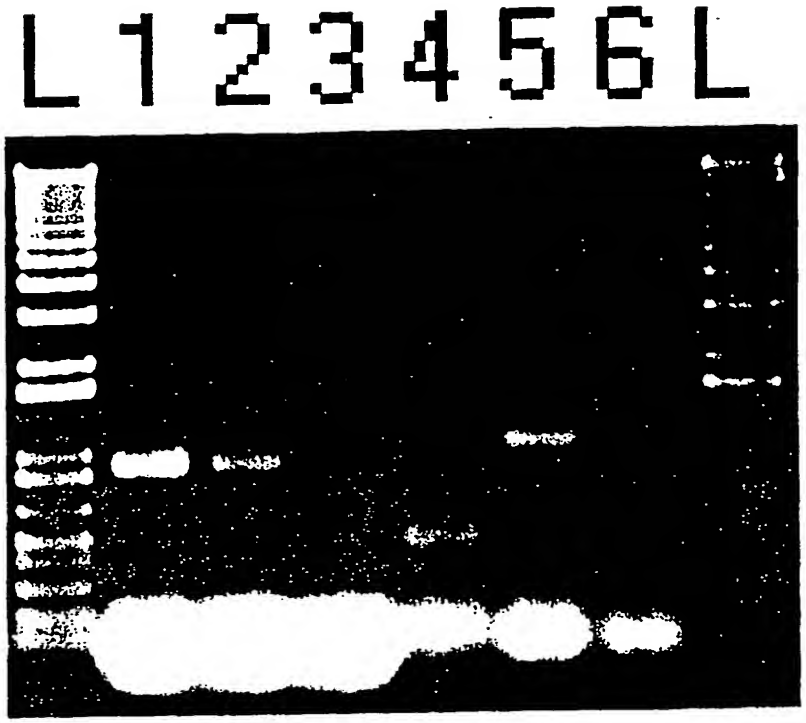


Fig 14

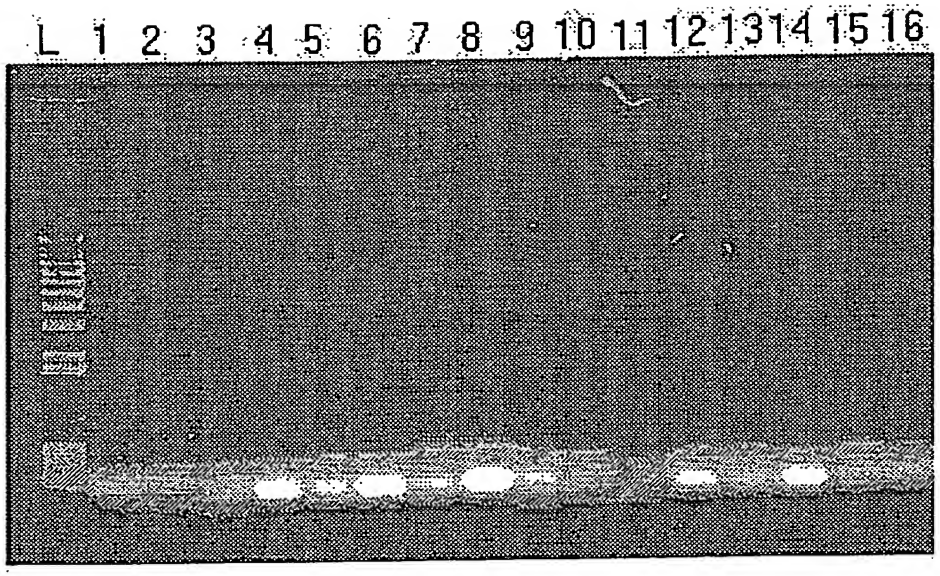


Fig 15

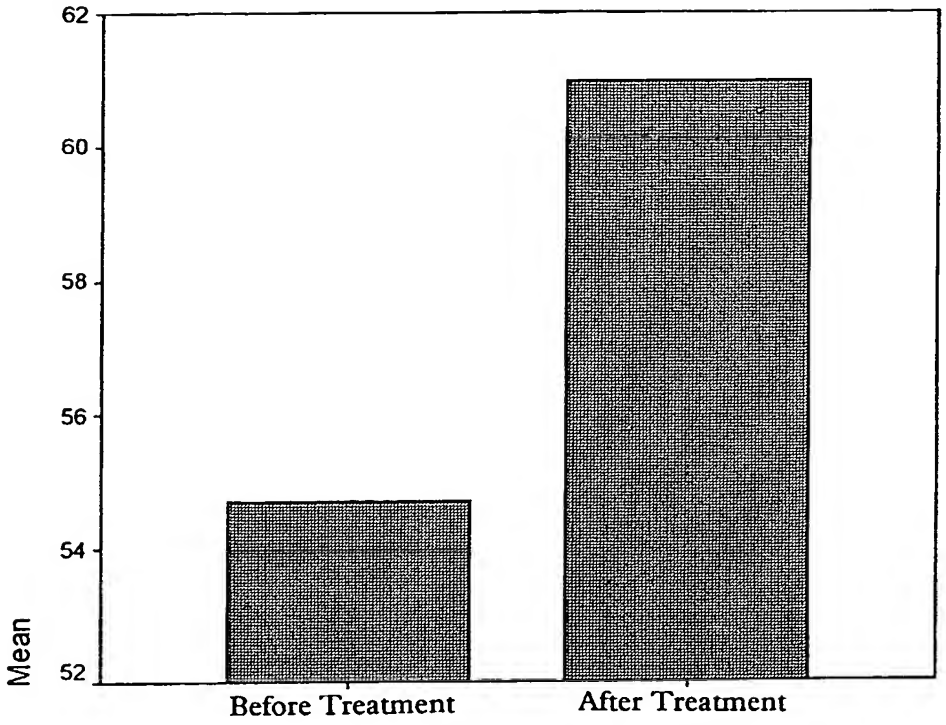

62
60
58
56
54
52
Mean
Before Treatment
After Treatment
Symptoms

Fig. 16

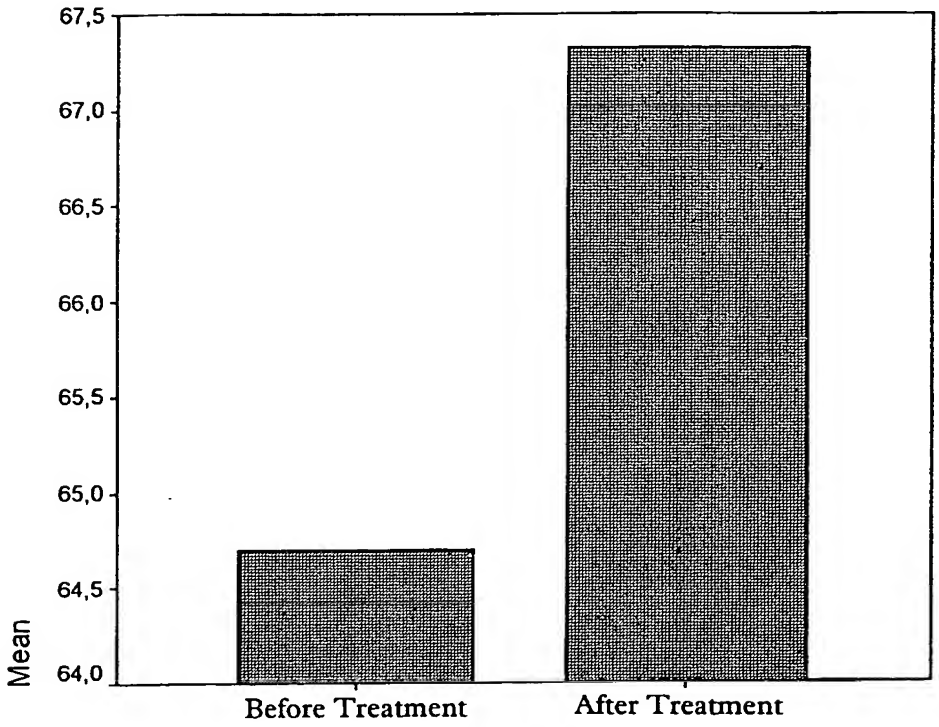

67,5
67,0
66,5
66,0
65,5
65,0
64,5
64,0
Mean
Before Treatment
After Treatment
Daily Activity

Fig. 17

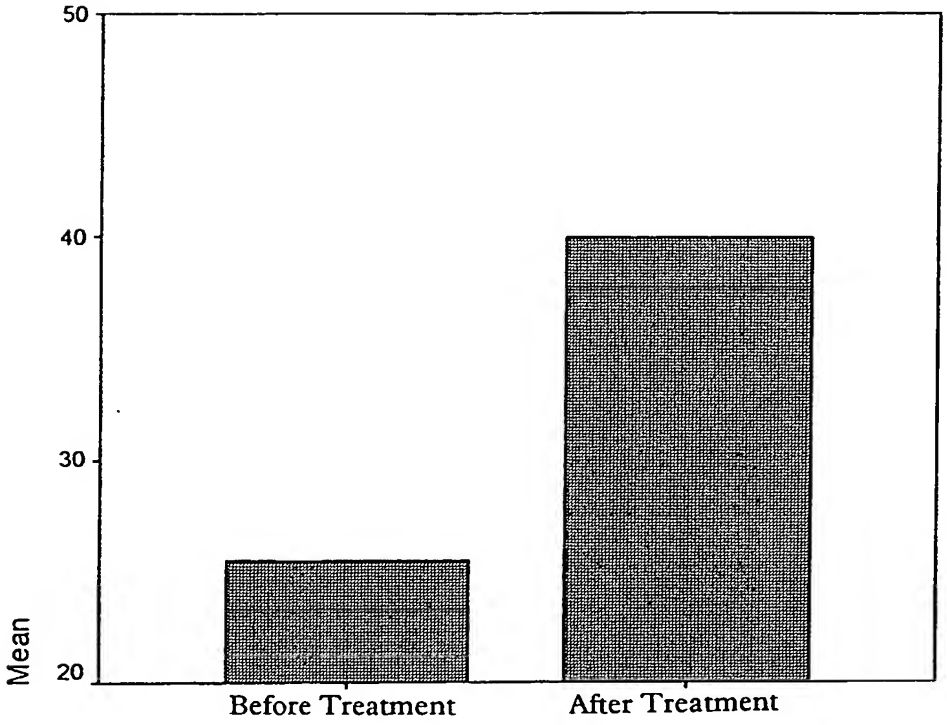
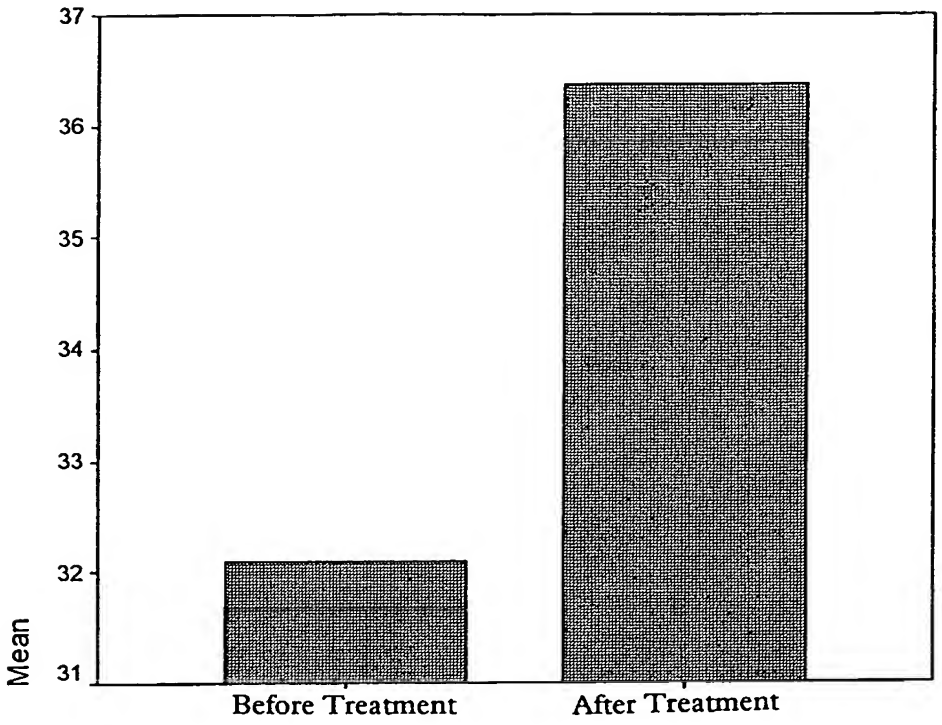

50
40
30
20
Mean
Before Treatment
After Treatment
Sport and Recreation

Fig. 18



Quality of Life

Fig. 19

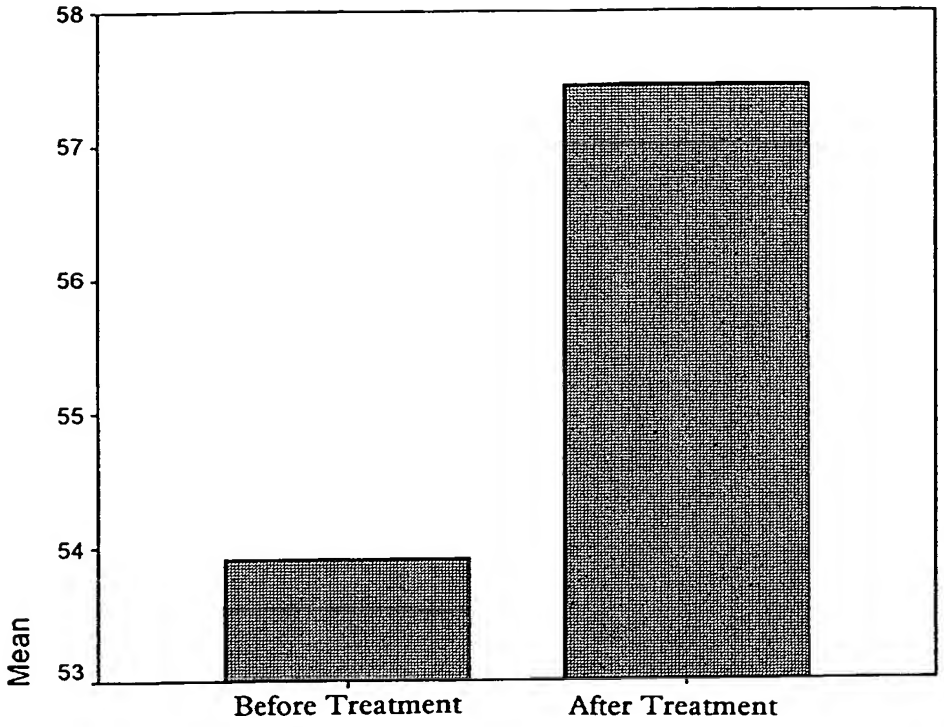
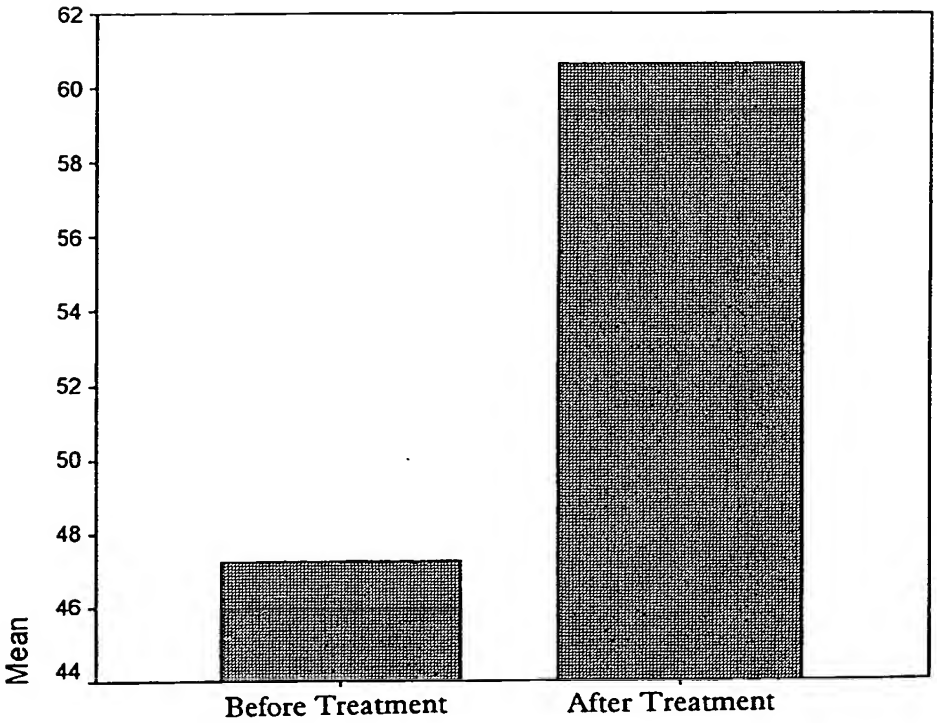


Fig. 20



Lysholm

Fig. 21